

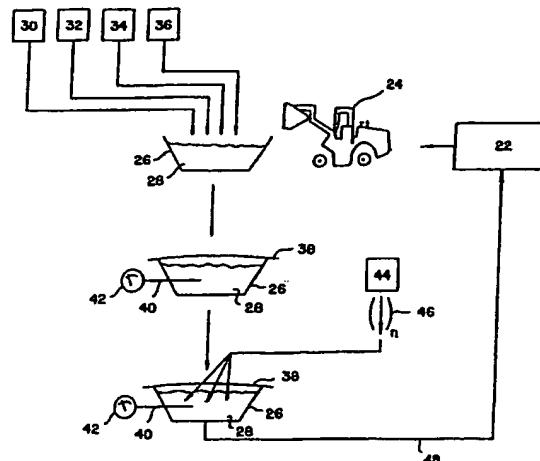
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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<p>(21) International Application Number: <b>PCT/US91/02416</b></p> <p>(22) International Filing Date: <b>9 April 1991 (09.04.91)</b></p> <p>(30) Priority data: 508,056 11 April 1990 (11.04.90) US</p> <p>(71) Applicant: IDAHO RESEARCH FOUNDATION, INC. [US/US]; 121 Sweet Avenue, P.O. Box 9645, Moscow, ID 83843-0178 (US).</p> <p>(72) Inventors: CRAWFORD, Donald, L. ; 1988 Appaloosa Road, Moscow, ID 83843 (US). STEVENS, Todd, O. ; 2455 George Washington Way, Apt. F130, Richland, WA 99352 (US). CRAWFORD, Ronald, L. ; 1812 Appaloosa Road, Moscow, ID 83843 (US).</p>		<p>(74) Agents: KLITZKE, Ramon, A., II et al.; Klarquist, Sparkman, Campbell, Leigh &amp; Whinston, One World Trade Center, Suite 1600, 121 S.W. Salmon Street, Portland, OR 97204 (US).</p> <p>(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), SE (European patent), SU.</p> <p>Published  <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>	
<p>(54) Title: <b>BIOLOGICAL SYSTEM FOR DEGRADING NITROAROMATICS IN WATER AND SOILS</b></p> <p>(57) Abstract</p> <p>Novel methods for biodegrading nitroaromatic compounds present as contaminants in soil or water using microorganisms are disclosed. Water is treatable directly; dry soil is first converted into a fluid medium by addition of water. The preferred method comprises two stages, each employing microorganisms: a fermentative stage, followed by an anaerobic stage. The fermentative stage is rapid, wherein an inoculum of aerobic and/or facultative microorganisms ferments a carbohydrate added to the fluid medium, exhausting the oxygen in the fluid medium and thereby inhibiting oxidative polymerization of amino by-products of the nitroaromatics. In the subsequent anaerobic stage, an inoculum of a mixed population of anaerobic microorganisms completes the mineralization of the contaminant nitroaromatics, using the remaining carbohydrate as a carbon and energy source. Preferably, the carbohydrate is a starch and the aerobic and/or facultative microorganisms are amylolytic, which cleave the starch at a moderate rate throughout both stages, ensuring a sustained supply of metabolizable carbohydrate. The microorganisms are preferably selected to be resistant to the types and concentrations of nitroaromatics present as contaminants.</p>			



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BIOLOGICAL SYSTEM FOR DEGRADING  
NITROAROMATICS IN WATER AND SOILS

Field of the Invention

This invention relates to the biodegradation of  
5 various nitroaromatic compounds in water and soils,  
including dinoseb (2-(1-methylpropyl)-4,6-dinitrophenol),  
using microorganisms.

Background of the Invention

Certain nitrophenolic compounds are sufficiently  
10 toxic to life to render them effective for use as  
herbicides, insecticides, or miticides. Such compounds  
include dinoseb (2-(1-methylpropyl)-4,6-dinitrophenol)  
which has been widely used as a herbicide since the 1950's  
on a variety of crops in the United States. Concerns for  
15 the safety of agricultural workers has resulted in  
discontinued use of dinoseb. However, numerous sites  
remain contaminated with this compound.

Other nitroaromatic compounds are similar to  
dinoseb in terms of chemical structure, but have other  
20 applications, such as in explosives. Such compounds  
include trinitrotoluene (TNT) and dinitrotoluene (DNT).  
Because of the widespread use of these compounds over a  
lengthy period of time, many sites have become  
contaminated with these compounds, including both  
25 manufacturing and military sites.

Many nitroaromatic compounds are either poorly  
degradable or nondegradable in field environments outside  
the laboratory. Previously, land farming was the favored  
method for disposing of these and other chemicals, wherein  
30 the chemicals were mixed with soil, fertilizer was added,  
and the mixture aerated to promote microbial activity.  
Unfortunately, nitroaromatics were not satisfactorily  
degraded by land farming or other well-aerated processes.  
Possible reasons include lack of nitroaromatic-degrading  
35 microorganisms, partitioning of the contaminant chemicals  
to biologically sequestered or inhospitable parts of the  
environment, and accumulation of toxic partial-breakdown  
by-products. Problems with land farming in general

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included the slow rate of biodegradation, high expense, and accumulation of toxic by-products.

Other methods have been used to remove nitroaromatics and similar compounds from contaminated soils, but with little practical success. Such methods include transportation of contaminated soil to hazardous waste dumps, and on-site incineration of the soil. Problems with such methods include high cost and poor accountability of the responsible party.

10 Previous laboratory studies indicated that certain nitroaromatic molecules are susceptible at least to microbiological transformation. However, the studies did not disclose biochemical mechanisms of such transformation or degradation or whether the nitroaromatic 15 compounds were completely mineralized. In one study, for example, a soil Moraxella microorganism was isolated that was capable of growth on p-nitrophenol as its only source of carbon and energy. Spain et al., Biochem. Biophys. Res. Comm. 88:634-641 (1959). In another study, the 20 anaerobic bacteria Veillonella alkalescens reductively transformed nitroaromatic compounds, converting the nitro groups to amino groups. McCormack et al., Appl. Environ. Microbiol. 31:949-958 (1976).

Aminoaromatic derivatives of nitroaromatics can 25 undergo enzymatic oxidation to form polymeric (large molecular weight) materials. Parris, Residue Revs. 76:1-30 (1980). In the field, such polymers are usually incorporated into soil humic matter. Channon et al., Biochem. J. 38:70-85 (1944); McCormick et al., Appl. Environ. Microbiol. 31:949-958 (1976); Simmons et al., Environ. Sci. Technol. 23:115-121 (1989). Humic matter tends to be long-lived in soils, thereby representing a major long-term environmental fate of many nitroaromatics and aminoaromatics. Other soil microorganisms are capable 35 of cleaving the azo linkages of polymerized aminoaromatics, often forming toxic by-products.

Bacteria are also able to attack nitrobenzoic acid, Cartwright and Cain, Biochem. J. 71:248-261 (1959),

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as well as o-nitrophenol and m-nitrophenol, Zeyer and Kearney, J. Agric. Food Chem. 32:238-242 (1984), where the nitro group is released as nitrite. Again, however, complete mineralization has not been demonstrated.

- 5 Further, nitrite release has not been found to be a significant pathway for highly substituted nitroaromatics. No instance is currently known where a compound possessing more than one nitro substituent has been completely mineralized. In fact, the pertinent literature presents  
10 no evidence supporting ring cleavage of highly substituted nitroaromatics. Kaplan, "Biotransformation Pathways of Hazardous Energetic Organo-Nitro Compounds," in Biotechnology and Degradation. Adv. Appl. Biotechnol. Ser. 4:155-181, Gulf Pub. Co., Houston, TX (1990).

- 15 Aromatic groups in general appear to be degradable via only a few aerobic and anaerobic pathways. Gottschalk, Bacterial Metabolism, 2d ed., Springer Verlag, New York (1986), pp. 157-162; Berry et al., Microbiol. Rev. 51:43-59 (1987); Schink, "Principles and Limits of  
20 Anaerobic Degradation: Environmental and Technological Aspects," in Zinder (ed.), Biology of Anaerobic Microorganisms, Wiley, New York (1988). Aerobically, many aromatic groups are degraded to catechol, protocatechuate or homogentisate by the action of oxygenase and  
25 dioxygenase enzymes. Catechol and protocatechuate can be degraded further by aromatic ring cleavage either ortho or meta to the hydroxyl groups. Because of the difficulty of working with anaerobic microorganisms and processes, biochemical pathways describing anaerobic degradation of  
30 aromatic compounds have been less well characterized.

- Alkyl groups on aromatic rings are degradable via reactions similar to those for simple alkanes. Under aerobic conditions, the terminal carbon is oxidized to yield a carboxylic acid. Degradation then proceeds  
35 by  $\beta$ -cleavage to yield either benzoates (odd-numbered carbon chains) or phenylacetates (even-numbered carbon chains). No anaerobic microorganisms capable of carrying out this process have been isolated to date. In spite of

the above results known in the art, there is little information currently available on practical means of using microbial cultures to bioremediate nitroaromatic-contaminated soils.

- 5       Dinoseb, an intensely yellow-colored compound visible at concentrations as low as 10 ppm, has been found to not significantly accumulate in agricultural soil at normal application rates, even after years of repeated application. Doyle et al., J. Agric. Food Chem.
- 10      26:987-989 (1978). However, higher application rates, such as from spills of substantial amounts of the compound, can result in appreciable accumulation at a site. Presumably, therefore, dinoseb at lower concentrations is transformed by certain soil
- 15      microorganisms. Such transformation appears to result only in the formation of amino and acetoamido forms of dinoseb, which apparently retain significant toxicity. Parris, Residue Revs. 76:1-30 (1980).

- 20      Previous work on the biotransformation of the explosive 2,4,6-trinitrotoluene (TNT) indicates that the primary mode involves transformation (reduction) of the nitro groups. Kaplan, supra. A recent paper from Soviet researchers describes degradation of TNT by a strain of Pseudomonas fluorescens. Naumova et al., Mikrobiologiya
- 25      57:218 (1988). But, while these reports shed some light on microbial events and hypothetical biochemical mechanisms therefor, they neither disclose nor suggest effective methods for bioremediating soils or wastewater contaminated with these compounds. Further, the Soviet
- 30      results have not been confirmed outside the U.S.S.R.

- Hence, although several anaerobic microbiological systems have been described for degrading other aromatic chemicals, little to no information is available on practical means of using these cultures to bioremediate
- 35      contaminated soils and waters, especially soils and waters contaminated with nitroaromatics. In today's world, effective remediation of environmental sites contaminated with compounds, such as nitroaromatics, requires that the

- 5 -

contaminants be completely mineralized to ensure the absence of latently toxic by-products. Such results for nitroaromatics simply have not been shown in the prior art, particularly as applicable to large-scale, low-cost  
5 bioremediation efforts.

Therefore, there remains a need for a method to effectively bioremediate dinoseb-contaminated soils, as well as soils contaminated with other nitroaromatic compounds, such as TNT and DNT.

10 Further, there is a need for such a method that can be performed at a natural site contaminated with dinoseb or a related nitroaromatic compound.

15 Further, there is a need for such a method that can completely degrade dinoseb and other nitroaromatics, leaving no detectable or environmentally significant amounts of aromatic by-products or other toxic intermediary compounds, including polymeric derivatives.

20 Further, there is a need for such a method employing microorganisms of types and species profiles normally found in many soil environments.

Further, there is a need for such a method that is inexpensive and easy to perform, particularly on a large-scale, in the field.

25 Further, there is a need for such a method that can be performed rapidly, including in the field.

Further, there is a need for such a method that will effect bioremediation of nitroaromatic-contaminated soil without specialized bioreactors or other complex equipment.

30 Summary of the Invention

In accordance with the present invention, soil or water contaminated with one or more nitroaromatic compounds is subjected to a two-stage bioremediation process employing different microorganisms during each  
35 stage. The stages comprise an initial fermentation stage followed by an anaerobic stage. Most of the actual biodegradation of the contaminant nitroaromatics takes place in the anaerobic stage. At the end of the anaerobic

stage, the contaminant nitroaromatics have been biodegraded to non-toxic end products.

As another aspect of the invention, complete biodegradation of nitroaromatics has been found to occur

5 only under anaerobic conditions. Since anaerobiosis generally requires an aqueous environment, it is usually necessary to add extraneous water to a nitroaromatic-contaminated soil to create a fluid mud slurry of the soil before beginning the process.

10 Contaminated water can be subjected to the process directly.

During the first stage of the process, the normally aerobic contaminated soil (with water added to form a mud slurry) or contaminated water alone is rendered 15 anaerobic. The preferred method for achieving an anaerobic condition is by a fermentation of a supply of starchy carbohydrate or other readily fermentable carbon source added to the slurry or water. Fermentation, where the carbon source is a starchy carbohydrate, is performed 20 by one or more species of aerobic or facultatively anaerobic amylolytic microorganisms inoculated into the slurry or water. Amylolytic microorganisms are not required if the carbon source is a simple sugar, such as fructose or glucose.

25 As another aspect of the invention, the aerobic or facultative microorganisms are preferably isolated and enriched in a culture containing the particular nitroaromatic present in the contaminated soil or water.

30 As another aspect of the invention, it is usually necessary at the beginning of the aerobic stage to add an extraneous source of nitrogen for the microorganisms. The nitrogen source is preferably in the form of ammonium ion or simple amino compounds readily utilizable by aerobic and anaerobic microorganisms.

35 As another aspect of the invention, it is preferable to stimulate a rapid onset of intense fermentation to quickly cause exhaustion of the oxygen dissolved in the slurry or water, thereby rendering the

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slurry or water anaerobic, without exhausting the carbon source. Quick attainment of anaerobiosis minimizes oxidative polymerization of any amino derivatives of the nitroaromatics that tend to form under aerobic conditions.

- 5 Once formed, such polymers are difficult to biodegrade. Rapid anaerobiosis can be achieved by inoculating the slurry or water with a large dosage of aerobic and/or facultative fermentative microorganisms.

If necessary, mineral nutrients, including phosphate salts, can be added to the soil slurry or water to facilitate microbial growth. Supplementary vitamins and cofactors may also be required, but probably only when treating wastewaters having very little dissolved organic carbon. Soils generally have sufficient nutrients.

- 10 As another aspect of the invention, the carbon source added to the soil slurry or water for aerobic fermentation is preferably a starchy carbohydrate substance hydrolyzable to constituent sugars by amylolytic microorganisms in the aerobic inoculum. A starch is  
15 preferred over merely adding free sugar because the starch serves as a reservoir of metabolizable carbohydrate that ensures an adequate supply of easily metabolizable sugar for the microorganisms, both in the aerobic stage and in the anaerobic stage. Anaerobic biodegradation of  
20 nitroaromatics requires such a sustained sugar supply. A supply of sugar added at the beginning of the aerobic stage would generally be exhausted prematurely, making it difficult to maintain anaerobic conditions for the  
25 requisite amount of time to achieve complete  
30 mineralization of the nitroaromatics.

As another aspect of the invention, the amount of starchy carbohydrate to be added to the volume of soil slurry or water to be treated can be "tailored" to ensure the desired degree of biodegradation is attained and waste  
35 of carbohydrate is avoided. The amount of carbohydrate should be just sufficient to supply the metabolic needs of the microorganisms until the anaerobic biodegradation of contaminant nitroaromatics is complete.

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As yet another aspect of the invention, once strict anaerobic conditions have been attained in the volume of slurry or water, an inoculum comprised of an anaerobic consortium of microorganisms is added to the  
5 volume to start the second, or anaerobic, stage.

Anaerobic conditions are preferably determined via a potentiometric measurement, where a redox potential of -200 mV or less indicates strict anaerobic conditions.

As yet another aspect of the invention, the  
10 anaerobic consortium comprises multiple species of microorganisms that have been grown in a medium containing one or more nitroaromatics identical or similar to the contaminant nitroaromatics to be biodegraded. The anaerobic microorganisms are able to biodegrade the  
15 nitroaromatics in the presence of metabolizable sugar to simple non-toxic compounds, such as methane, carbon dioxide, and acetate.

As yet another aspect of the invention, after inoculation, the anaerobic consortium is afforded  
20 sufficient time to biodegrade the contaminant nitroaromatics in the soil slurry or water to non-toxic end-products. Because degradation of the nitroaromatic compounds occurs in an anaerobic environment, polymerization to toxic humic-like compounds and other  
25 large, long-lived, latently toxic molecules normally formed in aerobic environments is prevented.

As yet another aspect of the invention, the present method is preferably performed in a suitably large covered vessel for containing the contaminated soil slurry or water during bioremediation. Such containment ensures that anaerobic conditions in the soil slurry or water are reached more rapidly and are better controlled and maintained. Containment also facilitates easier control of environmental parameters, such as temperature, pH, and,  
35 if needed, escape of volatile gases from the slurry or water being treated.

It is accordingly one object of the present invention to provide a method for effectively

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bioremediating soils and waters contaminated with one or more nitroaromatic compounds.

Another object of the present invention is to provide such a method that can be performed at natural 5 sites contaminated with nitroaromatics.

Another object of the present invention is to provide such a method that will allow contaminant nitroaromatics in soil or water to be biodegraded to such an extent that no detectable or environmentally 10 significant amounts of aromatic by-products or other toxic intermediary compounds are left in the soil or water, including latently toxic polymeric derivatives of the nitroaromatics.

Another object of the present invention is to provide such a method that utilizes microorganisms similar 15 to those found in many soil and aquatic environments.

Another object is to provide such a method that is inexpensive and easy to perform, particularly on a large scale in the field.

Another object is to provide such a method that can be performed rapidly, even in the field.

These and other objects, features, and advantages of the present invention will become apparent with reference to the following description and drawing.

25 Brief Description of the Drawing

The drawing consists of multiple figures, in which:

FIG. 1 is a schematic depiction of the formation of an amino derivative of the representative nitroaromatic 30 compound "dinoseb" under aerobic conditions and the subsequent aerobic polymerization of amino derivatives into undesirable humic-like compounds retaining the latent toxicity of dinoseb.

FIG. 2 is a graph showing the rate of 35 biodegradation of dinoseb according to the present invention as a function of temperature.

FIG. 3 is a graph showing the relationship to dinoseb biodegradation rate of various concentrations of

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sugar (carbon), and ammonium chloride (nitrogen) for the anaerobic consortium of microorganisms.

FIG. 4 is a graph showing the rate of biodegradation of dinoseb according to the present invention as a function of pH.

FIG. 5 is a graph showing the kinetics of anaerobic biodegradation of dinoseb by the anaerobic consortium to intermediary compounds and the subsequent disappearance of the intermediary compounds.

FIG. 6 is a graph similar to FIG. 5 showing the kinetics of anaerobic biodegradation of 4,6-dinitro-*o*-cresol by an anaerobic consortium previously acclimated to dinoseb.

FIG. 7 is a graph similar to FIG. 5 showing the kinetics of anaerobic biodegradation of 3,5-dinitrobenzoate by an anaerobic consortium previously acclimated to dinoseb.

FIG. 8 is a graph similar to FIG. 5 showing the kinetics of anaerobic biodegradation of 2,4-dinitrotoluene by an anaerobic consortium previously acclimated to dinoseb.

FIG. 9 is a graph similar to FIG. 5 showing the kinetics of anaerobic biodegradation of 2,6-dinitrotoluene by an anaerobic consortium previously acclimated to dinoseb.

FIG. 10 is a graph similar to FIG. 5 showing the kinetics of anaerobic biodegradation of dinitrophenol by an anaerobic consortium previously acclimated to dinoseb.

FIG. 11 is a schematic depiction of a reaction pathway for the anaerobic biodegradation of dinoseb according to the process of the present invention.

FIG. 12 is a graph showing the effect of BESA, an inhibitor of methanogenesis, on the anaerobic biodegradation of dinoseb by an anaerobic consortium.

FIG. 13 is another schematic depiction of a reaction pathway for the anaerobic biodegradation of dinoseb according to the process of the present invention.

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FIG. 14 is a representative schematic depiction of a process according to the present invention as it would be conducted at a field site.

Detailed Description of the Preferred Embodiment

5 I. Selection and Isolation of Dinoseb-Degrading Microorganisms

various microorganisms capable of at least transforming dinoseb were preliminarily selected and enriched using a chemostat having a 1-L capacity vessel  
10 and agitation capability (Series 500 Fermenter, L. H. Fermentation, Hayward, CA). An approximately 250 mL volume of 3 mm diameter glass beads were placed in the bottom of the chemostat vessel to act as a soil-holding matrix. As a result, both aerated (supernatant liquid)  
15 and non-aerated (soil sediment) enrichment conditions were simultaneously maintained in the chemostat.

As used herein, "transformation" of dinoseb or other nitroaromatic compound means a chemical change other than degradation. The simplest way to confirm  
20 transformation of dinoseb is to observe the disappearance of the bright yellow color of the compound. Under aerobic conditions, dinoseb is transformed into an amino form that subsequently becomes polymerized by oxidative coupling. The term "degradation" denotes the complete mineralization  
25 of the subject nitroaromatic to methane, carbon dioxide, and acetate.

To provide a source of mineral nutrients to the microorganisms in the chemostat, a mineral nutrient solution was prepared containing the following solutes:  
30  $\text{KH}_2\text{PO}_4$  (272 mg/L),  $\text{K}_2\text{HPO}_4$  (348 mg/L),  $\text{Na}_2\text{SO}_4$  (5 mg/L),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (5 mg/L),  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (1 mg/L), and  $\text{FeSO}_4$  (0.5 mg/L). The nutrient solution was supplemented with selected carbon and nitrogen sources, as discussed further below, in an attempt to effect a satisfactory preliminary  
35 selection of dinoseb-degrading microorganisms. The initial inoculum of microorganisms consisted of indigenous microbes associated with a 200-gram sample of a soil mixture removed from a site previously exposed to dinoseb

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and suspected of having dinoseb-degrading activity. The chemostat was operated at a flow rate of 10 mL/hr, pH 7, and 25°C. Carbon and nitrogen sources for the microorganisms used individually in separate attempts at 5 selection included: 50 ppm dinoseb plus 50 ppm 2,4-dinitrophenol, 50 ppm dinoseb plus 50 ppm 2,4-dinitrophenol plus 1 g/L NH<sub>4</sub>Cl, 50 ppm dinoseb plus 50 ppm phenol, and 100 ppm dinoseb plus 0.5 g/L glucose and 1 g/L NH<sub>4</sub>Cl.

10 No dinoseb degradation or turbidity occurred in the chemostat during thirty days' incubation and agitation with 50 ppm dinoseb plus 50 ppm 2,4-dinitrophenol as the sole carbon substrate. When the chemostat was operated for another thirty days with 50 ppm dinoseb plus 50 ppm 15 2,4-dinitrophenol and 1 g/L NH<sub>4</sub>Cl, no dinoseb degradation or turbidity resulted. When the chemostat was operated for forty-two days with 50 ppm dinoseb, 50 ppm 2,4-dinitrophenol, and 50 ppm phenol, no dinoseb degradation occurred, but turbidity did develop. Most 20 importantly, when the chemostat was operated with 0.5 g/L glucose, 100 ppm dinoseb, and 1 g/L NH<sub>4</sub>Cl, turbidity developed immediately and dinoseb degradation began after twenty days. The latter result indicated that, to effect degradation of dinoseb by either aerobic or anaerobic 25 processes, supplementary glucose is required as a carbon source. Continuing with the latter operational conditions, the flow rate was then increased to 20 mL/hr for another thirty days to complete the preliminary selection process.

30 After preliminary selection, samples of the supernatant liquid presumed to contain various aerobic and/or facultative microorganisms were removed from the chemostat and plated on nutrient agar to obtain a number of isolates of specific microorganisms. The isolates were 35 then cultured under aerobic, anaerobic, and microaerophilic conditions wherein their individual abilities to transform or degrade dinoseb were evaluated. For aerobic culturing, the mineral nutrient solution

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described above was used, supplemented with the following:  
dinoseb (50 mg/L), glucose or fructose (500 mg/L), NH<sub>4</sub>Cl  
(1.0 g/L), MnCl<sub>2</sub> 4H<sub>2</sub>O (0.5 mg/L), H<sub>3</sub>BO<sub>3</sub> (0.05 mg/L), ZnCl<sub>2</sub>  
(0.05 mg/L), CuCl<sub>2</sub> (0.03 mg/L), Na<sub>2</sub>MoO<sub>4</sub> 2H<sub>2</sub>O (0.01 mg/L),  
5 CoCl<sub>2</sub> 6H<sub>2</sub>O (0.5 mg/L), NiCl<sub>2</sub> 6H<sub>2</sub>O (0.05 mg/L), Na<sub>2</sub>SeO<sub>3</sub> (0.05  
mg/L), and a vitamin solution recommended by Wolin et al.,  
J. Biol. Chem. 238:2882-2886 (1963). Yeast extract was  
added to the culture solution to a concentration of  
0.5 g/L. Yeast extract served as a convenient source of  
10 additional carbon and energy for the microorganisms, as  
well as a source of additional vitamins and cofactors.

For culturing under denitrifying (anaerobic) conditions, the aerobic culture medium was supplemented with 1 g/L KNO<sub>3</sub>, boiled under nitrogen gas, and sealed in  
15 glass containers with butyl rubber stoppers before inoculation. Culturing under denitrifying conditions is a way of selecting for facultative anaerobes. Under such conditions, nitrate is employed by the respiring microbes as an electron acceptor rather than oxygen as used by  
20 respiring aerobic microorganisms. Facultative anaerobes can be cultured in either aerobic or anaerobic environments.

A reduced anaerobic mineral medium (RAMM) was used for culturing the microorganisms under anaerobic  
25 conditions. RAMM comprised the same ingredients as listed above for aerobic cultures, but with 10 mg/L resazurin added as a redox indicator, 10 mg/L Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> 2H<sub>2</sub>O added as a reducing agent, and 1.2 g/L NaHCO<sub>3</sub>. (Preferably, 0.1 g/L yeast extract is also added.) Anaerobic cultures were  
30 grown in serum bottles and balch tubes sealed with butyl rubber stoppers, using strict anaerobic procedures, as detailed in Ljungdahl and Wiegel, "Working with Anaerobic Bacteria," in Demain and Solomon (eds.), Manual of Industrial Microbiology and Biotechnology, American  
35 Society for Microbiology, Washington, D.C. (1986)  
pp. 84-96.

Several isolates of microorganisms which could aerobically transform dinoseb were obtained from the

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supernatant liquid of the chemostat containing glucose, dinoseb, and NH<sub>4</sub>Cl. Characteristics of these isolates are presented in Table 1.

Table 1

5 Isolates That Aerobically Transform Dinoseb

	<u>Isolate</u>	<u>Gram Reaction</u>	<u>Shape</u>	<u>Colony Morphol.<sup>a</sup></u>	<u>Oxidase</u>	<u>Catalase</u>	<u>Facultative</u>	<u>Dinoseb Transformation When:</u>
10	TDN-1	+	rods	R,O,P	+	+	no	yes
	TDN-2	-	rods	I,T	+/-	+	yes	yes
	TDN-3	-	rods	R,T	-	+	yes	yes <sup>b</sup>
15	TDN-4	+/-	rods	R,Y	+	+	yes	yes
	TDN-5	+/-	cocci	S,T	+/-	+	yes	yes

<sup>a</sup> = Colonial Morphologies: R = round, I = irregular, S = spreading, T = transparent, O = opaque, Y = yellow, P = produces yellow-green pigment.

<sup>b</sup> = Dinoseb transformation in this strain is inhibited by nitrate

Under microaerophilic conditions, every strain caused the dinoseb-containing culture medium to turn bright red. Such transformation was not noted with any of the strains in aerated dinoseb-containing cultures. As used herein, the term "microaerophilic conditions" refers to a culture environment having an appreciably depressed concentration of dissolved oxygen as compared to aerobic conditions, but not so low as to be strictly anaerobic. Under such conditions, aminoaromatics can still undergo polymerization reactions. This is in contrast to strictly anaerobic conditions under which such polymerization reactions are blocked. Microorganisms termed "microaerophiles" undergo optimal growth under microaerophilic conditions.

The red substance obtained under microaerophilic conditions with all the strains could not be extracted with organic solvents, nor could it be resolved by thin-layer chromatography (TLC). Analysis confirmed that the red substance was the amino derivative of dinoseb. After two to three weeks, the red color faded and a brown precipitate formed. TLC analysis of the brown precipitate showed a continuous smear with no discernable bands. These results indicate that dinoseb is transformed under microaerophilic conditions to an amino form which is

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oxidatively polymerized to larger random-length molecules and not degraded.

Strains TDN-2 and TDN-5 appeared to be facultative anaerobes and were able to carry out the 5 transformation of dinoseb to the red metabolite under denitrifying (anaerobic) conditions, in which case the brown precipitate did not form.

To quantify the transformation of dinoseb by the strains of Table 1, dinoseb concentrations were determined 10 by High Performance Liquid Chromatography (HPLC) using a binary gradient of 10% tetrahydrofuran and methanol (solution A) and 1% acetic acid and water (solution B) on a 250 x 2 mm Phenomenex "Spherex" 5  $\mu$ m C18 reverse-phase column (Phenomenex Corp., Rancho Palos Verdes, CA). A 15 Hewlett-Packard Model 1090A instrument, equipped with a diode-array detector and a computerized data system, was used for the analyses. The solvent flow rate was 0.4 mL/min, and the column temperature was 40°C. The gradient program was a ten-minute gradient from 60% solution A plus 20 40% solution B to 100% solution A, followed by five minutes at 100% solution A. Detection of dinoseb and transformation products thereof was by use of the diode-array detector, measuring UV absorption at 268, 225, and 385 nm, with continuous scanning of the absorption 25 spectrum from 190 to 450 nm.

HPLC analysis of the culture medium from strains TDN-2 and TDN-5, of Table 1 also showed accumulation of a single dinoseb-transformation product with a HPLC retention time of 2.05 minutes. Dinoseb transformation by 30 strain TDN-3 was inhibited by nitrate in both microaerophilic and denitrifying conditions. No dinoseb transformation occurred in anaerobic cultures of TDN-4, but transformation did occur when the flasks were opened and the cultures exposed to air. Strain TDN-1 was 35 obligately aerobic and only transformed dinoseb under microaerophilic conditions.

As indicated in Table 1, these isolates of dinoseb-transforming microorganisms were taxonomically

diverse, although no definite species identifications were made. Despite this apparent species diversity, the isolates appeared to carry out similar reactions when transforming dinoseb.

5       The above results indicate that, in oxygen-containing environments, the isolates obtained from the aerobic supernatant of the chemostat only reduced the nitro groups of dinoseb, thereby forming amino products that were subsequently oxidized by extracellular enzymes  
10      to form amorphous polymeric compounds, as diagrammed in FIG. 1. Since the dinoseb is apparently not actually degraded via such an aerobic process, but merely "transformed" into an amorphous polymer, the products of the process probably latently retain all the toxicity of  
15      dinoseb. Thus, it appears that aerobic bacteria such as certain of the strains listed in Table 1 do not actually detoxify dinoseb and would, therefore, not be appropriate for use in bioremediation of nitroaromatic contaminants in wastewaters or contaminated soils.

20       Although the above results were useful in elucidating the mechanism of dinoseb transformation in aerobic environments, it became clear that a biological method for degrading dinoseb and related microaromatic compounds from contaminated soils and waters must include  
25      the use of microorganisms selected in a dinoseb-containing anaerobic environment. As a result, microorganisms resident in the anaerobic sediment of the chemostat supplied with medium containing 100 ppm dinoseb plus 0.5 g/L glucose and 1 g/L NH<sub>4</sub>Cl were cultured and tested.

30       A consortium (stable mixed population) of anaerobic microorganisms capable of degrading dinoseb to non-aromatic products was enriched from the population of such organisms in the chemostat as follows. Sediment from the chemostat consisting of soil was used to inoculate  
35      strictly anaerobic medium comprised of the mineral nutrient solution described above with added 1 g/L fructose, 1 g/L NH<sub>4</sub>Cl, and 100 ppm dinoseb. After five weeks' incubation, the bright yellow color of the medium

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(due to the presence of dinoseb) changed to a bright orange, then faded to colorlessness followed by development of turbidity. This activity could be maintained in mineral medium for three or four 5 sediment-free transfers, but not in medium containing 0.2 g/L yeast extract or 5% rumen fluid. Sediment-free anaerobic dinoseb-degrading cultures could be maintained indefinitely by making three transfers in mineral medium, followed by one transfer in yeast extract-containing 10 medium, followed by further mineral medium transfers. After eighteen months of such transfers, the dinoseb-degrading cultures remained stable in the yeast-extract containing medium, which resulted in 5-to 15 10-fold faster degradation of dinoseb than in mineral medium without yeast extract.

Degradation of dinoseb to non-aromatic products by the anaerobic consortium did not occur unless strict anaerobic procedures were followed during preparation of the media and during culture transfers. This indicates 20 that actual dinoseb degradation to non-aromatic products, as opposed to mere transformation, is an anaerobic process.

Several parameters should be controlled for optimal dinoseb degradation by the anaerobic consortium. 25 These include temperature, nitrogen and pH.

As shown in FIG. 2, the optimal temperature for dinoseb degradation is about 25°C. Although FIG. 2 only covers a range from 20°C to 40°C, a useful temperature range would be within the range for mesophilic 30 microorganisms, generally between 10°C and 40°C. Temperatures higher than about 40°C would either kill important microorganisms or shut down key enzymatic reactions. Lower temperatures within this range, including temperatures within the range 10°C to 20°C, 35 would merely result in a slower metabolic rate of the microorganisms, the rate generally dropping by about half for every ten-degree drop in temperature.

As shown in FIG. 3, the optimal sugar concentration (either fructose, glucose, or other simple sugar is suitable) is about 0.5 g/L, as indicated by the "carbon" line. The optimal NH<sub>4</sub>Cl concentration is about 5 4 g/L, as indicated by the "nitrogen" line. However, the anaerobic culture is largely insensitive to NH<sub>4</sub>Cl concentration between about 1 and 5 g/L. NH<sub>4</sub>Cl serves as an important source of nitrogen for the anaerobic microorganisms. Other nitrogen-containing compounds can 10 also be used, so long as the nitrogen is in a form such as ammonium ion or amino groups. The nitrogen source cannot be a nitrate because nitrates inhibit the process of nitroaromatic degradation by these microorganisms.

Finally, the optimal pH is about 7, as shown in 15 FIG. 4, but the anaerobic culture appears to be largely insensitive to pH values between 6 and 8.

The anaerobic consortium degraded dinoseb via a series of intermediate aromatic products (A, B, C, and D, as shown in FIG. 5) which could be detected by HPLC. As 20 determined by UV absorption spectra, no further aromatic products could be detected after thirty days, indicating that complete cleavage of the aromatic ring occurred. Such aromatic cleavage is a key step in the degradation of dinoseb to non-toxic compounds.

25 The stable consortium of anaerobic microorganisms contained at least three bacterial morphologies, including: short coccobacilli, 1-1.5  $\mu\text{m}$  long; medium-sized rods, .75x2  $\mu\text{m}$ ; and large rods, 1-1.5x4  $\mu\text{m}$ . Briefly exposing the consortium to air before anaerobic 30 incubation resulted in elimination of the large rods. Also, dinoseb degradation did not proceed beyond intermediate D (FIG. 5). When the anaerobic consortium was used to inoculate dinoseb-containing aerobic media, a single bacterial species grew. That species was a 35 gram-negative rod which grew as a coccobacillus under anaerobic conditions. The coccobacillus transformed dinoseb to a single uncharacterized product in aerobic cultures and to a different product in anaerobic cultures.

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The bacterium also was catalase positive, oxidase negative, and most closely matched Klebsiella oxytoca (similarity index 0.677), using the BioLog GN identification system (Hayward, CA). The BioLog GN 5 identification system is a method of testing bacteria for utilization of ninety-five different carbon substrates, where the resulting pattern growth is compared with a database of patterns for known species of bacteria.

The anaerobic consortium was tested for its 10 ability to degrade other nitroaromatic compounds. Utilization of other nitroaromatic substrates was determined by growing cultures in medium similar to that described above, but with 50 ppm of the appropriate nitroaromatic compound substituted for dinoseb. The 15 anaerobic consortium was able to completely degrade 4,6-dinitro-*o*-cresol (DNOC, FIG. 6) and 3,5-dinitrobenzoate (3,5-DNB, FIG. 7) to non-aromatic compounds. 2,4-dinitrotoluene (2,4-DNT, FIG. 8) and 2,6-dinitrotoluene (2,6-DNT, FIG. 9) were degraded to 20 intermediate products, but it was unclear whether or not the products were eventually degraded to non-aromatics. After sixty days, the concentration of 2,4-dinitrophenol (2,4-DNP, FIG. 10) began to decline, but the parent compound persisted for at least four months in these 25 cultures. Degradation of these other nitroaromatic compounds was much slower than for dinoseb.

A dinoseb anaerobic degradation pathway consistent with the above results is shown in FIG. 11.

When bromoethanesulfonic acid (BESA), a specific 30 inhibitor of methanogenesis, was added to the anaerobic consortium at 200  $\mu$ M concentration, dinoseb degradation was slowed and products C and D accumulated (FIG. 12). These products remained in the culture medium for at least another three months. Accumulation of C indicated that D 35 is formed from C and that BESA at least partially blocked formation of D. Therefore, the reaction from C to D is probably a hydrogen-generating reaction. Since the

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reaction results in an increase in hydrophobicity, it probably involves removal of one or both acetamide groups.

The anaerobic dinoseb-degradation intermediates were tentatively identified as follows. Anaerobic dinoseb

5 cultures were extracted and subjected to TLC, as described above, during both the early stages (orange color) and later stages (colorless) of dinoseb degradation. Extracts from the early stages yielded two TLC bands which were not present in uninoculated controls. GCMS analysis indicated  
10 that both bands contained multiple compounds. Similar analysis of the extract from the later stages of degradation yielded one band not present in uninoculated controls. The mass spectra showed fragmentation patterns similar to that for dinoseb, confirming that they  
15 correspond to dinoseb derivatives. The intermediates as identified are shown in FIG. 11.

The molecular formulas of the intermediates, based on isotope abundance calculations from the molecular weights (+/- 0.005 AMU), are shown in Table 2. The  
20 intermediate with molecular weight 220 could not be assigned a molecular formula; however, the stated mass was expected from 2-sec-butyl-4-nitro-6-aminophenol. The major product in the later stages of degradation was identified as 2-aminobenzoic acid (anthranilic acid) by  
25 GCMS comparison with authentic standards.

Table 2  
Accurate Mass Determinations and Molecular Formulas for Compounds Detected by GC/MS

	<u>source</u>	<u>molecular mass</u>	<u>standard deviation</u>	<u>molecular formula</u>	<u>deviation from observed mass</u>
30	dinoseb	240.0699	.0084	C <sub>10</sub> H <sub>12</sub> N <sub>2</sub> O <sub>5</sub>	.0047
35	band 1	210.0891	.0063	NR	
	band 1	220.0998	.0081	NR	
	band 1	264.0995	.0065	NR	
40	band 2	234.0966	.0071	C <sub>12</sub> H <sub>14</sub> N <sub>2</sub> O <sub>3</sub>	.0038
	band 2	165.1147	.0046	C <sub>10</sub> H <sub>15</sub> NO	.0007

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band 1B	136.060	.0088	C <sub>7</sub> H <sub>8</sub> N <sub>2</sub> O	.0035
	band 1B	137.0508	.0047	C <sub>7</sub> H <sub>7</sub> NO <sub>2</sub>

5 NR = no reasonable formula could be calculated from this mass reading

Under anaerobic conditions the reduced dinoseb derivatives would not be subject to the oxidative coupling reactions found in the aerobic environment. Partially 10 degraded molecules persisting as dissolved monomers are, therefore, available for further biodegradation by various anaerobic bacteria.

It is unclear why an external carbon source is required for dinoseb mineralization by the anaerobic 15 consortium of microorganisms. One or more of these microorganisms may effect certain chemical changes to the dinoseb molecule, but derive no metabolic energy from such reactions or from the products of such reactions.

Alternatively, microorganisms that carry out later steps 20 in the degradation process might be inhibited by dinoseb. In the latter case, ring cleavage products would not be available for use by bacteria carrying out early steps in the degradation process.

The results described above indicate, therefore, 25 that dinoseb is biodegradable by the anaerobic consortium of microorganisms. The initial step involving reduction of nitro groups to amino groups appears to occur in a manner similar to that for rumen microorganisms.

Intermediates detected having molecular weights of 220, 30 234, and 264 were not identified, but probably represent N-alkylated forms of reduced dinoseb. The alkyl side chain of dinoseb is also anaerobically removed, as indicated by formation of anthranilic acid. FIG. 13 shows a dinoseb degradation pathway consistent with the results 35 above.

Studies in which [<sup>14</sup>C]-ring-labeled dinoseb was degraded as above yielded [<sup>14</sup>C]-acetate, indicating that dinoseb rings are cleaved. Additionally, we have found that the presence of nitroaromatics inhibits

methanogenesis. However, the nitro groups are readily reduced to amino groups, after which methanogenesis resumes. The only intermediates identified to date are amino compounds, but numerous other "intermediates" have 5 been found that remain to be specifically identified.

When tested with other nitroaromatic substrates, the anaerobic consortium was able to completely degrade nitrocresols or nitrobenzoates, but was unable to cleave the aromatic ring of nitrotoluenes or to degrade 10 nitrophenols. It is probable that other selections performed in a manner similar to that described above would give rise to other anaerobic populations which can utilize these compounds. For example, passing a medium containing a nitrophenolic compound through a chemostat, 15 as described above, inoculated with a diverse population of soil microorganisms would be expected to select for anaerobes capable of mineralizing nitrophenols. In other words, anaerobic consortia can be "tailored" for degrading a particular type of nitroaromatic compound.

20 II. Bioremediation of Fluid Media

As used herein, the term "fluid medium" refers to waters and slurries, including mud, comprising water plus soil or other particulate material.

The process of the present invention requires an 25 aqueous fluid medium because anaerobiosis can only occur in aqueous environments. As a result, bioremediation of a contaminated dry soil requires that water be added, forming a mud slurry, to provide a sufficiently aqueous environment for metabolic activity by the anaerobic 30 consortium of microorganisms. Such an aqueous environment is automatically provided when the process is to be applied to a contaminated wastewater.

In addition, the anaerobic consortium is unable 35 to withstand prolonged exposure to oxygen. Consequently, an inoculum comprised of such microorganisms should not be added to a volume of soil slurry or wastewater to be subjected to biodegradation without first rendering the volume anaerobic. The transition to anaerobiosis should

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be as rapid as possible to preclude aerobic oxidative coupling of the contaminant nitroaromatics to polymeric forms via the action of indigenous aerobic microorganisms.

For the experiments described below, the

- 5 anaerobic dinoseb-degrading consortium isolated as described above was maintained on a reduced anaerobic mineral medium (RAMM) consisting of:  $\text{KH}_2\text{PO}_4$  (0.27 g/L),  $\text{K}_2\text{HPO}_4$  (0.35 g/L),  $\text{NH}_4\text{Cl}$  (1.5 g/L), glucose (0.5 g/L), yeast extract (0.1 g/L),  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (15 mg/L),  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  (20 mg/L),  $\text{FeCl}_2 \cdot 2\text{H}_2\text{O}$  (4 mg/L),  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  (0.5 mg/L),  $\text{H}_3\text{BO}_3$  (0.05 mg/L),  $\text{ZnCl}_2$  (0.05 mg/L),  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (0.05 mg/L),  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$  (0.05 mg/L),  $\text{CuCl}_2$  (0.03 mg/L),  $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$  (0.01 mg/L),  $\text{NaHCO}_3$  (2.4 g/L), and 1 ng/L resazurin.
- 10 Cultures were incubated in darkness without shaking at 15  $30^\circ\text{C}$ . Strict anaerobic procedures were followed during all media preparations and transfer operations. Ljungdahl and Wiegel, "Working with Anaerobic Bacteria," in Demain and Solomon (eds.), Manual of Industrial Microbiology and Biotechnology, American Society for Microbiology,
- 15 Washington, D.C. (1986).

In order to sustain anaerobiosis in the present process, a source of readily-metabolizable carbon, such as sugar, is required as a supplement. Preferably, the carbon source is a complex carbohydrate from which sugars 25 are "released" over a period of time, rather than a supply of sugar that is completely "available" for immediate consumption. Because complex carbohydrates must be enzymatically cleaved to yield metabolizable sugar, they allow maintenance of strict anaerobic conditions in the 30 fluid medium to be extended for a time period sufficient to biodegrade the particular concentration of contaminant nitroaromatic without the need to add more carbon source. For example, if a large amount of an "available" sugar, such as glucose or fructose, were added to an aerobic 35 aqueous slurry of soil, anaerobiosis would be quickly achieved. However, maintenance of strict anaerobiosis for the several weeks that may be required to achieve complete degradation of the nitroaromatic would be difficult due to

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an initially very high rate of sugar metabolism followed by premature exhaustion of the sugar supply.

Although sugar could be added at one or more additional times during maintenance of anaerobic

- 5 conditions before complete nitroaromatic degradation was achieved, the added sugar would have to be added in controlled amounts at specific times and mixed each time into the fluid being treated. Such adding and mixing impart unnecessary complexity to the method. Further, use  
10 of more complex carbohydrates, such as starch, as a carbon source results in a steadier degradation rate. Repeated additions of sugar result in an undulating rate which is less efficient.

- In view of the above, desired characteristics of  
15 the carbon source include a high energy content, ready metabolizability, low numbers of indigenous heterotrophic bacteria which might compete with the anaerobic inoculum, and sustained metabolic availability sufficient to maintain prolonged steady rate of anaerobiosis. On the  
20 basis of these criteria, a hydrolyzable polysaccharide such as starch is a more suitable carbon source than free sugar.

After evaluation of a number of starchy by-products from various food processing plants, a  
25 preparation of dewatered solids from a potato processing plant was selected as the preferred carbon source. The characteristics of the potato waste included: 42% solids, 215 mg/g available starch, 6.7 mg/g total nitrogen,  $2.6 \times 10^4$  culturable heterotrophic bacteria per gram, and  
30  $8 \times 10^3$  culturable amylolytic (able to hydrolyze starch) bacteria per gram. Chief advantages of starchy potato waste are available in large amounts and at a low cost. However, any vegetable or grain starch would probably suffice.

- 35 Candidate starchy carbohydrates were analyzed for solid content by weighing after oven drying, and for total nitrogen content by the Kjeldahl procedure commonly known in the art. Available starch was determined by incubating

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sterilized 1-gram samples with 300 units of  $\alpha$ -amylase and 100  $\mu$ L of diazyme L-100 (Miles Pharmaceuticals, Elkhart, IN) for twenty-four hours in 10 mL of sterile 0.4 M phosphate buffer, pH 7.0. After incubation, the 5 samples were diluted to 100 mL and analyzed for reducing sugars using the dinitrosalicylate assay with similarly prepared glucose as a standard. Miller, Anal. Chem. 31:426-428 (1959). Available starch was assayed as mg sugar released per gram dry weight of starchy 10 carbohydrate. Numbers and types of bacteria in starchy carbohydrates were determined by standard plate counts in aerobic mineral medium agar containing (for total heterotrophic counts) 2 g glucose, 0.4 g yeast extract, 1 g  $\text{NH}_4\text{Cl}$ , and 1 g  $\text{NaNO}_3$  per liter, or (for amylolytic 15 bacteria) 2 g soluble starch, 1 g  $\text{NH}_4\text{Cl}$ , and 1 g  $\text{NaNO}_3$  per liter for amylolytic bacteria.

Since the anaerobic consortium cannot withstand prolonged exposure to air, it is necessary to pretreat the volume of wastewater or soil slurry with a rapid aerobic 20 fermentation step to deplete oxygen in the volume and achieve anaerobiosis before adding the anaerobic consortium. The transition from aerobic fermentation to anaerobiosis should be as rapid as possible to preclude oxidative coupling of aminoaromatic derivatives of the 25 contaminant nitroaromatic in the aerobic step. According to the results of tests performed using loamy sand and rich silt-loam soils, simply flooding the soil with water does not produce sufficiently rapid anaerobiosis. Mere addition of sugar produces rapid anaerobiosis by an 30 initial high rate of aerobic fermentation that rapidly consumes the available oxygen in the liquid. However, as discussed above, use of sugar as a carbon source instead of a complex carbohydrate, such as starch, usually results in premature exhaustion of the carbon source. Also, even 35 with elevated concentrations of added sugar, at least partially aerobic conditions often reform before completion of the nitroaromatic degradation. Use of dewatered potato solids or other starchy carbohydrate that

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is metabolized more slowly than free sugar results in a satisfactorily rapid anaerobiosis following aerobic fermentation, while yielding a sustained sugar concentration over the period of time required to

5 completely degrade the contaminant nitroaromatic.

To evaluate the relative effectiveness of starchy carbohydrates as a carbon and energy source versus a sugar such as glucose alone, 300-g samples of various types of soil, such as loamy sand and rich silt-loam, were

10 individually placed in 1-L Erlenmeyer flasks and flooded with 200 mL of 0.4 M phosphate buffer (pH 7) to form a mud slurry. Flasks were covered with aluminum foil and incubated without shaking at 25°C. At various times during incubation, both pH and redox potential of the  
15 slurry were measured potentiometrically at 0, 1, and 3 cm beneath the liquid surface. A 1 mL sample was also removed for each analysis of residual sugar. A redox potential of -200 mV or less was indicative of a strictly anaerobic condition in the liquid.

20 The amount of starchy carbohydrate to add to a volume of wastewater or mud slurry for particulate dosages of fermentative and anaerobic organisms may have to be determined experimentally and optimized for a particular soil or wastewater and the particular type and  
25 concentration of contaminant nitroaromatic. To reduce costs in the actual bioremediation process, the amount of starch should be determined that will just sustain anaerobic conditions for the time required to achieve the desired degree of nitroaromatic degradation and no longer.

30 Any of a number of species of fermentative amylolytic microorganisms indigenously present in the starchy carbohydrate can serve to hydrolytically cleave the starch into constituent sugars when the starch is added to a volume of wastewater or slurry of soil. Such  
35 fermentative microorganisms would include aerobic and/or facultative microorganisms. Starch cleavage occurs via amylase enzymes secreted by the microorganisms into the surrounding aqueous medium. Although amylase action is

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optimal in aerobic environments, the enzyme will continue to hydrolyze starch when the medium becomes anaerobic. Additionally, many facultative amylolytic microorganisms will survive and continue to secrete amylase after a 5 medium has been converted from aerobic to anaerobic. If sugar is used as the carbon source, the fermentative microorganisms need not be amylolytic.

In a series of incubation tests where the soil contained 25 ppm dinoseb, anaerobiosis occurred only after 10 several days in the presence of the starchy potato by-product, where the initial aerobic fermentation was performed by fermentative amylolytic bacteria (representing a number of species) indigenous to the starchy potato by-product. Several days to achieve 15 anaerobiosis is too long. Evidently, the indigenous bacterial population was adversely stressed by the dinoseb and unable to metabolically respond in a rapid manner. To selectively enrich for dinoseb-resistant amylolytic bacteria, additional starchy potato by-product and dinoseb 20 (to 100 ppm) were added to a flask from these experiments. After a prolonged incubation to achieve selection, several dinoseb-resistant bacteria were isolated from the flask. One strain (strain DSA-1), identified as facultative 25 Klebsiella oxytoca by the BioLog GN system, retained good amylolytic activity in the presence of 100 ppm dinoseb.

When dinoseb-containing soil received an inoculum of Klebsiella oxytoca strain DSA-1, along with the starchy potato by-product, a greatly reduced time to achieve anaerobiosis was noted. In the latter case, anaerobiosis 30 was established almost as fast as in control soils lacking dinoseb. Klebsiella oxytoca strain DSA-1 was thus regarded as a preferred inoculation strain with which to achieve anaerobiosis for degrading dinoseb in the presence of a starchy carbohydrate.

35 Although Klebsiella oxytoca strain DSA-1 was the particular bacterium selected for in the above-described experiments involving dinoseb, it is expected that other selections performed in a similar manner using a source of

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starch containing indigenous aerobic and/or facultative "fermentative" microorganisms will probably yield other species and strains after selection. Additionally, it is expected that using a nitroaromatic other than dinoseb for 5 supplying the selection pressure would probably give rise to other satisfactory fermentative species and/or strains resistant to the particular nitroaromatic. The lesson from these studies is that the indigenous microflora associated with starches are probably not sufficiently 10 tolerant to most nitroaromatics to facilitate the required high initial rate of fermentation to achieve rapid anaerobiosis in a volume of soil slurry or wastewater containing nitroaromatic compounds. As a result, it will probably be necessary to employ an aerobic or facultative 15 species and strain preselected against the particular nitroaromatics to be biodegraded.

In order to achieve anaerobiosis in the shortest amount of time, it is preferable to inoculate using a large dose of preselected fermentative microorganisms. 20 Our studies indicate that a dose of about  $10^7$  to  $10^8$  CFU per gram dry soil or mL water is an effective dose. Cost constraints generally preclude larger doses. Also, larger doses generally do not achieve proportionately shorter times to anaerobiosis.

25 In another series of tests, bioremediations of 1-kg samples of each type of dinoseb-contaminated soil (loamy sand and silt-loam) were individually performed by adding water, starchy potato by-product, and pretreating the resulting slurry with a large inoculum (as above) of 30 Klebsiella oxytoca strain DSA-1. After each slurry was rendered anaerobic (redox potential -200 mV), it was inoculated with a similarly large dose of the dinoseb-degrading consortium of anaerobic microorganisms. Dinoseb was found to be completely converted to 35 intermediate compounds within one week of anaerobic inoculation. After four weeks, the concentrations of the intermediate compounds declined below detectable limits. At the end of the four-week period, only 0.5 ppm of

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dinoseb could be Soxhlet-extracted from the treated loamy sand soil, and none could be extracted from the treated silt loam. By contrast, in the uninoculated loamy sand control, dinoseb did not decline. In the uninoculated 5 silt loam control, dinoseb did decline after several days' lag time, but no intermediate compounds metabolically derived from dinoseb were detected.

In similar experiments, soils contaminated with like concentrations of 4,6-dinitro-o-cresol and 10 3,5-dinitrobenzoate were bioremediated within thirty days.

To perform the above experiments, soils were adjusted to 100 ppm dinoseb before adding any microorganisms or starchy carbohydrate by adding to the soil a solution of dinoseb in methanol and allowing the 15 soil to dry completely afterward. For each soil sample, 1 kg dinoseb-supplemented soil was mixed with 2 g of the starchy carbohydrate. The mixture was inoculated with the amylolytic bacteria, placed in an open two-liter capacity erlenmeyer flask, and a sufficient volume 0.4 M phosphate buffer (pH 7) was added to saturate the water-holding 20 capacity of the soil. When the redox potential of the soil solution became less than -200 mV, a 50 mL volume (O.D.  $\approx$  1) of the anaerobic consortium was injected below the surface of the soil. Samples (approximately 5 g each) 25 were removed periodically, weighed and vortexed with 5 mL 0.1 N NaOH. Samples were centrifuged to remove the solids, and the supernatant was analyzed for dinoseb and its metabolites. At the end of the incubation, the remaining soil was extracted and analyzed for dinoseb.

Dinoseb concentrations were determined by high 30 performance liquid chromatography (HPLC) using a binary gradient of 10% tetrahydrofuran in methanol (solution A) and 1% acetic acid in water (solution B) on a 250x2 mm Phenomenex "Spherex" 5  $\mu$ m C18 reverse-phase column. 35 Analyses were performed using a Hewlett-Packard Model 1090A instrument, as described above. Dinoseb was extracted from soils by Soxhlet extraction with ethyl acetate for five hours. Before extraction, each sample

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was amended with 200  $\mu$ L of a 0.25% solution of 4,6-dinitro-o-cresol in methanol, which served as an extraction standard, and lyophilized. Samples were then amended with 100  $\mu$ L of 0.01 M H<sub>2</sub>SO<sub>4</sub>. After extraction,  
5 the extracts were dried over anhydrous NaSO<sub>4</sub>, evaporated under a vacuum, and dissolved in 5 mL of ethyl acetate.

Parallel experiments utilizing [<sup>14</sup>C]-dinoseb were performed to evaluate mass-balance relationships for dinoseb and its degradation products. Details are  
10 described below. Results are shown in Table 3.

Table 3

Percent of Total Radioactivity

	Treatment	CO <sub>2</sub>	Polar Extract	Nonpolar Extract	Solid	Percent Recovery
15	Loamy Sand Inoculated	29.6	43.3	5.11	4.0*	82
20	Loamy Sand Uninoculated	0.3	0.8*	100.8	6.7*	108
	Silt Loam Inoculated	32.1	29.9	3.5	8.9*	74
25	Silt Loam Uninoculated	0.8	1.1*	31.3	44.8	78

\* = these values were not significantly different from background counts at the 90% confidence level

Referring to Table 3, about 30% mineralization to [<sup>14</sup>C]-CO<sub>2</sub> was obtained after thirty days' incubation of samples of inoculated soils, as compared to less than 1% for the uninoculated controls. Most of the remaining radioactivity in the inoculated soils was present as polar metabolites. Less than 10% of the radioactivity was associated with solids in the inoculated soils after extraction, as compared to nearly 45% in the silt-loam control. In the loamy sand control, virtually all of the radioactivity was associated with the nonpolar extract,  
35 which contained undegraded dinoseb. In addition, although methane was not quantified in these experiments, some radioactivity appears to end up as methane.  
40

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To perform the radiochemical studies described above, radiolabeled dinoseb (u-ring [<sup>14</sup>C]-dinoseb) was synthesized from [<sup>14</sup>C]-phenol and was 96% radiochemically pure, as determined by HPLC analysis and TLC coupled with liquid scintillation counting. After adding 1  $\mu$ Ci u-ring [<sup>14</sup>C]-dinoseb to the soil, the flasks were stoppered and a glass trap containing 1 mL of 1 M KOH was suspended in each flask. The KOH solution was exchanged daily during incubations, rinsed with 1 mL water and counted with 18 mL of BioSafe II liquid scintillation cocktail (RPI Inc., Mt. Prospect, IL). At the end of the incubation, each soil culture was connected to a CO<sub>2</sub> trap consisting of a series of four stoppered serum bottles each containing 10 mL of 1 N KOH. The gaseous effluent first passed through a C18 "Sep-Pak" cartridge (Waters Associates, Milford, MA) wetted with methanol to remove volatile organics. Each soil culture was then acidified with 10 mL concentrated HCl, agitated, and flushed with nitrogen gas to drive off dissolved CO<sub>2</sub>. One-mL samples from each trap were counted with 19 mL of BioSafe II.

Three 25-gram subsamples of each soil slurry were neutralized and extracted with 0.1 N NaOH. The extracts were neutralized, then passed through a "Sep-Pak" C18 cartridge which was rinsed with 1 mL water. The solid 25 subsamples were dried, Soxhlet extracted, and the extracts were pooled with ethylacetate elutions from the "Sep-Pak" cartridges. From both the polar and non-polar extracts, 1 mL samples were counted with 19 mL of BioSafe II. Finally, 0.25 gm of each extracted soil sample was mixed 30 with 19 mL BioSafe II and counted. All radioactive samples were counted in a Beckman Model 7000 liquid scintillation counter. A control flask for each soil type was treated similarly except that no bacterial inoculations were made.

35 In the above experiments, simply flooding the soils did not produce anaerobiosis in the resulting soil slurries, even in a slurry of the rich silt-loam soil. It is probable that anaerobiosis occurred within highly

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- localized sites within the slurry, such as soil pores, but this was not sufficient to support growth of the oxygen-sensitive anaerobic consortium. In every case, exogenous carbon, preferably as a starchy carbohydrate 5 metabolized by amylolytic microorganisms, was required to produce the requisite strict anaerobiosis that would allow growth of the subsequently added dinoseb-degrading anaerobic microorganisms.

The experiments described above show that

- 10 exogenous, strictly anaerobic microorganisms can be used to degrade nitroaromatic chemicals in soil or wastewater. The mass balance and  $^{14}\text{C}$  data indicate that the anaerobic consortium mediated a complete destruction of dinoseb as a representative nitroaromatic, rather than mere 15 polymerization of the compound, which occurred in the silt-loam soil in the absence of anaerobic inoculation.

- The method of the present invention, therefore, comprises basically two stages: an initial fermentative stage wherein the wastewater or aqueous slurry of 20 nitroaromatic-contaminated soil is fortified with a starchy carbohydrate and inoculated with "fermentative" (aerobic and/or facultative) amylolytic bacteria that hydrolyze the starch, metabolize a portion of the sugars produced by the hydrolysis, and consume the oxygen in the 25 liquid; and a subsequent anaerobic stage wherein the wastewater or aqueous slurry of contaminated soil is inoculated with a consortium of anaerobic bacteria that metabolize the remaining sugar and degrade the contaminant nitroaromatic. This bioremediation method may be used for 30 any soil or water contaminated with dinoseb or other nitroaromatic or aminoaromatic compounds subject to polymerization reactions in aerobic soil, such as 4,6-dinitro-*o*-cresol and 3,5-dinitrobenzoate.

- Although anaerobiosis can be performed in an open 35 environment, it is inefficient and difficult to control unless performed in a closed environment, particularly on a large scale. As a result, the method of the present

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invention is performed in the field, preferably in a suitably large liquid-containment vessel or the like.

FIG. 14 is a schematic representation of one embodiment of the process of the present invention, as it

- 5 would be conducted in the field to bioremediate a nitroaromatic-contaminated soil. In FIG. 14, the contaminated soil 22 is transferred via equipment 24 to a plastic-lined pit 26 or the like. After the soil 22 is added to the pit 26, starchy carbohydrate 30, a nitrogen source 32, water 34, and an inoculum of fermentative amylolytic microorganism 36 are added to the soil 22, forming a slurry 28 in the pit 26. After forming the slurry 28, a cover 38 is placed over the pit 26, where the plastic-lined pit 26 and cover 38 together serve to
- 10 exclude gaseous exchange between the slurry 28 and the atmosphere. The slurry 28 is monitored using a potentiometric probe 40 coupled to a readout 42 to enable one to determine when anaerobic conditions have developed in the slurry 28 (redox potential about -200 mV or less).
- 15 After the slurry 28 reaches anaerobiosis, an inoculum of an anaerobic consortium 44 of microorganisms is added to the slurry 28 beneath the surface, after performing sufficient numbers of serial transfers 46 of the anaerobic microorganisms 44 to yield a suitably large dose. The
- 20 slurry 28 is maintained in an anaerobic condition in the covered pit until the nitroaromatics are biodegraded, at which time the slurry 28 may be returned 48 to the original site.

- 25 The process of FIG. 14 is a batch process. In a batch process applied to nitroaromatic-contaminated wastewater (not shown), the contaminated water is simply conducted into the plastic-lined pit 26 of FIG. 14 or analogous vessel for bioremediation according to the present method. In the case of soil, it is usually necessary to add water to the soil to form a mud slurry, which is a more suitable environment for anaerobiosis than dry soil. Such a slurry will have a proportion of water relative to soil of about 15% to 20% (w/w) or more,

depending upon the type of soil and the degree of nitroaromatic contamination, to preferably bring the soil at least to about 100% water-holding capacity. More water can be added, if desired. More water added to the soil  
5 lessens the effective concentration of the contaminant nitroaromatic.

Since most soils and many natural waters already have a broad spectrum of mineral nutrients available to support life, in many cases it will not be necessary to  
10 add to the water or mud slurry many of the supplementary mineral nutrients required for laboratory cultures. In virtually all cases, however, an extraneous nitrogen source 32 will be required, such as ammonium chloride. Many soils and most waters will also require extraneous  
15 phosphate (not shown), such as sodium or potassium phosphate. The nitrogen and phosphate requirements for the present process can be satisfied in many cases by merely adding ammonium phosphate (a common "fertilizer") as a supplement until the approximate desired  
20 concentration of ammonium and phosphate are attained. Since each soil and water is different, preliminary analysis of the soil or water may be needed to determine indigenous concentrations of ammonium and phosphate so that the proper amount of supplementary ammonium and  
25 phosphate can be determined so as to avoid waste.

As stated above, the nitrogen source 32 should not be nitrate because nitrates inhibit the ability of the anaerobic microorganisms to biodegrade nitroaromatics.

It may be necessary, particularly in the case of  
30 strongly acidic waters or soils contaminated with nitroaromatics, to adjust the pH of the fluid medium to within the preferred range of about 6 to 8. An inexpensive additive, such as lime, is satisfactory for elevating the pH. In most cases, however, significant pH  
35 adjustment will not be necessary. Extrapolating from the graph of FIG. 4, it would seem that pH values appreciably above or below (especially above) the preferred range of 6

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to 8 would not cause a catastrophic depression of microbial metabolism required for bioremediation.

The preferred target concentrations of key nutrients (including contributions by the soil and/or

5 water) are as given in the list of such ingredients in the nutrient solutions used in the studies described above.

The ammonium concentration, as stated earlier, should preferably be between 0.4% and 0.5% (w/v), but a concentration between 0.1% and 0.6% (w/v) would suffice.

10 The overall ionic strength of the water or slurry to be treated should preferably be approximately that of the laboratory cultures of microorganisms, but the microbes are tolerant to surprisingly large variations of ionic strength.

15 The temperature should preferably be between about 10°C and 40°C, with the optimal temperature about 25°C. As discussed above, lower non-freezing temperatures tend to slow microbial metabolism and correspondingly increase the length of time required to achieve  
20 bioremediation.

While yeast extract is an important supplement for laboratory cultures, it is not necessary in virtually all field applications, since soils and wastewater often contain "vitamins," cofactors, and other products of  
25 natural organic processes utilizable by the microorganisms of the present process.

The inoculum of fermentative amylolytic microorganisms 36 should be added at a dose of  $10^7$  to  $10^8$  CFU (colony forming units) per gram dry soil or per mL  
30 water. Higher doses are usually not necessary. Lower doses may result in a longer-than-optimal time to reach anaerobiosis. In addition, an amount of starchy carbohydrate 30, determined as described above, is also added. Preferably, the amount of starchy carbohydrate  
35 added per unit amount of water or soil slurry is experimentally "tailored" to sustain anaerobiosis for a sufficient amount of time to achieve the desired degree of nitroaromatic degradation in the water or soil. Since

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types and concentrations of nitroaromatic contaminants will differ among various soils and waters, and since soils and waters themselves will differ, the optimal amount of starchy carbohydrate required will probably 5 differ at each of various contaminated sites.

While the specific anaerobic consortium 44 of microorganisms described herein is suitable for degrading dinoseb and certain other nitroaromatics as described, other consortia of anaerobic microorganisms selected for 10 and isolated in a manner as described herein, but using a nitroaromatic other than dinoseb may be more suitable for other nitroaromatics.

Although employing fermentative microorganisms to render the water or slurry anaerobic is the preferred 15 method, other methods may be employed (not shown). However, it is anticipated that other methods may be prohibitively expensive. Such other methods include purging oxygen from the liquid using, for example, nitrogen or argon gas. However, gas-purging is typically 20 slower and less efficient in achieving satisfactory anaerobic conditions than employing aerobic fermentation. Another method would require adding oxygen-scavenging agents (strong reducing agents) to the liquid. Although use of reducing agents may be efficient (and rapid), the 25 disadvantage is that such agents represent other contaminants added to the water or slurry. As a result, use of microbial fermentation of sugar or starch is the preferred approach for achieving anaerobiosis.

After the contaminant nitroaromatic has been 30 satisfactorily biodegraded in the volume of water or slurry contained in the covered pit 26 or vessel, the pit 26 or vessel may be drained and a new volume of contaminated water or mud slurry added for bioremediation. In a semicontinuous process, about 10% to 15% of the 35 previous batch of treated water or slurry may be left in the pit 26 or vessel to aid in the inoculation of the subsequent batch. Additionally, such a semicontinuous process would preferably be controlled by various

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continuous electronic and chemical monitoring techniques known in the art, such as of dissolved oxygen and specific ions, as well as environmental conditions such as pH and temperature. Concentrations of nitroaromatic contaminants 5 and their metabolic intermediaries can be discontinuously monitored using HPLC and gas chromatography, for example.

Having illustrated and described the principles of our invention with reference to detailed descriptions of process steps and specific examples, it should be 10 apparent to those of ordinary skill in the art that the invention may be modified in arrangement and detail without departing from such principles. We claim as our invention all such modifications as come within the true spirit and scope of the following claims.

CLAIMS

1. A method for at least partially degrading nitroaromatic compounds present as contaminants in a fluid medium to reduce the concentrations of the nitroaromatic compounds in the fluid medium, the method comprising:
  - (a) adding to the fluid medium an inoculum of fermentative microorganisms resistant to the types and respective concentrations of contaminant nitroaromatic compounds present in the fluid medium, where the fermentative microorganisms are selected from the group consisting of aerobic and facultative microorganisms;
  - (b) adding to the fluid medium a carbohydrate fermentable by said fermentative microorganisms;
  - (c) maintaining the fluid medium containing said inoculum of fermentative microorganisms and carbohydrate under conditions suitable for growth of said fermentative microorganisms in the fluid medium, accompanied by fermentation by said microorganisms of a portion of said carbohydrate, thereby depleting the concentration of dissolved oxygen in the fluid medium, so as to render the fluid medium an aerobic;
  - (d) after the fluid medium has been rendered anaerobic, adding to the fluid medium an inoculum of anaerobic microorganisms capable of utilizing the carbohydrate as carbon and energy source and degrading said nitroaromatic compounds; and
  - (e) maintaining anaerobic conditions in the fluid medium for a time sufficient for the anaerobic microorganisms to degrade the contaminant nitroaromatic compounds.
2. A method for degrading nitroaromatic compounds as recited in claim 1, including the step of adding mineral nutrients to the fluid medium to support growth of the microorganisms inoculated into the fluid medium.
3. A method for degrading nitroaromatic compounds as recited in claim 1, including the step of adding a source of metabolizable nitrogen to the fluid

medium to support the growth of the microorganisms inoculated into the fluid medium.

4. A method for degrading nitroaromatic compounds as recited in claim 3, wherein the source of 5 metabolizable nitrogen is ammonium chloride.

5. A method for degrading nitroaromatic compounds as recited in claim 4, wherein the ammonium chloride is added to the fluid medium to yield a concentration of ammonium chloride in the fluid medium 10 within a range of about 4 mg/L to 5 mg/L.

6. A method for degrading nitroaromatic compounds as recited in claim 1, wherein the fluid medium is maintained at a pH within a range of about 6 to 8.

7. A method for degrading nitroaromatic 15 compounds as recited in claim 1, wherein the fluid medium is maintained at a temperature within a range of about 10°C to less than about 40°C.

8. A method for degrading nitroaromatic compounds as recited in claim 1, wherein said anaerobic 20 microorganisms are capable of degrading dinoseb present as a contaminant in the fluid medium.

9. A method for degrading nitroaromatic compounds as recited in claim 8, wherein the inoculum of fermentative microorganisms includes Klebsiella oxytoca 25 strain DSA-1.

10. A method for degrading nitroaromatic compounds as recited in claim 9, wherein said Klebsiella oxytoca strain DSA-1 microorganisms are resistant to dinoseb at a concentration of up to at least 100 ppm.

30 11. A method for degrading nitroaromatic compounds as recited in claim 1, wherein the fermentative microorganisms are added at an inoculum dosage within a range of at least about  $10^7$  to  $10^8$  CFU per gram of fluid medium.

35 12. A method for degrading nitroaromatic compounds as recited in claim 1, wherein said anaerobic microorganisms are capable of degrading nitrotoluene compounds present as contaminants in the fluid medium.

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13. A method for degrading nitroaromatic compounds as recited in claim 12, wherein said nitrotoluene compounds present as contaminants in the fluid medium include trinitrotoluene.

5 14. A method for degrading nitroaromatic compounds as recited in claim 1, wherein formation of polymerized derivatives of said contaminant nitroaromatic compounds in the fluid medium is inhibited.

10 15. A method for degrading nitroaromatic compounds as recited in claim 1, wherein the inoculum of fermentative microorganisms is comprised of a population of microorganisms grown in aerobic nutrient medium including the nitroaromatic compounds present as contaminants in said fluid medium.

15 16. A method for degrading nitroaromatic compounds as recited in claim 1, wherein the redox potential of the fluid medium when anaerobic is less than about -200 mV.

20 17. A method for degrading nitroaromatic compounds as recited in claim 1, wherein the carbohydrate is comprised of a starch.

25 18. A method for degrading nitroaromatic compounds as recited in claim 17, wherein said carbohydrate is comprised of a dewatered potato by-product.

19. A method for degrading nitroaromatic compounds as recited in claim 17, wherein the fermentative microorganisms are amylolytic.

30 20. A method for degrading nitroaromatic compounds as recited in claim 1, wherein the inoculum of anaerobic microorganisms is comprised of a population of anaerobic microorganisms grown in nutrient medium including the nitroaromatic compounds present as contaminants in said fluid medium.

35 21. A method for degrading nitroaromatic compounds as recited in claim 1, wherein the inoculum of anaerobic microorganisms is a consortium of multiple species of anaerobic bacteria.

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22. A method for degrading nitroaromatic compounds as recited in claim 21, wherein the inoculum of anaerobic microorganisms is comprised of at least three different species of anaerobic bacteria.

5 23. A method for degrading nitroaromatic compounds as recited in claim 1, wherein at least a portion of said nitroaromatic compounds is degraded to carbon dioxide.

10 24. A method for degrading nitroaromatic compounds as recited in claim 1, including the steps of placing the nitroaromatic-contaminated fluid medium in a covered vessel before rendering the fluid medium anaerobic and keeping the fluid medium contained within the covered vessel until said anaerobic microorganisms have been added  
15 to the vessel and have degraded the nitroaromatic compounds in the fluid medium.

20 25. A method for remediating a soil so as to at least partially reduce the concentration of nitroaromatic compounds present as contaminants in the soil, the method comprising:

(a) adding water to the nitroaromatic-contaminated soil to form an aqueous slurry of the soil;

25 (b) adding to the slurry an inoculum of fermentative amylolytic microorganisms resistant to the types and concentrations of nitroaromatic compounds present in the soil, where the fermentative amylolytic microorganisms are selected from the group consisting of aerobic and facultative amylolytic microorganisms;

30 (c) adding to the slurry at least one type of starchy carbohydrate hydrolyzable to constituent sugars by said fermentative amylolytic microorganisms;

35 (d) maintaining the slurry containing said inoculum of fermentative amylolytic microorganisms and starchy carbohydrate under conditions suitable for growth of said aerobic microorganisms in the slurry, accompanied by hydrolysis of the starchy carbohydrate to constituent sugars and metabolism by said microorganisms of a portion

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of said sugars, thereby depleting the concentration of dissolved oxygen in the slurry so as to render the slurry anaerobic;

- (e) after the slurry has been rendered  
5 anaerobic, adding to the slurry an inoculum of anaerobic microorganisms capable of utilizing as a carbon and energy source said sugars formed in the slurry by the amylolytic microorganisms and capable of degrading said nitroaromatic compounds; and  
10 (f) maintaining anaerobic conditions in the slurry for a time sufficient for the anaerobic microorganisms to degrade the contaminant nitroaromatic compounds.

26. A method for remediating soil as recited in  
15 claim 25, wherein the water is added to the soil in a volume sufficient to saturate the water-holding capacity of the soil.

27. A method for remediating soil as recited in claim 25, including the step of adding a source of  
20 metabolizable nitrogen to the slurry to support the growth of the microorganisms inoculated into the slurry.

28. A method for remediating soil as recited in claim 26, wherein the source of metabolizable nitrogen is ammonium chloride.

25 29. A method for remediating soil as recited in claim 27, wherein the ammonium chloride is added to a concentration in the water within a range of about 4 mg/L to about 5 mg/L.

30. A method for remediating soil as recited in claim 25, wherein the slurry is maintained at a pH within a range of about 6 to 8.

31. A method for remediating soil as recited in claim 25, wherein the slurry is maintained at a temperature within a range of about 10°C to less than  
35 about 40°C.

32. A method for remediating soil as recited in claim 25, wherein the inoculum of fermentative amylolytic microorganisms is comprised of a population of

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microorganisms grown in aerobic nutrient medium including the nitroaromatic compounds present as contaminants in said soil.

33. A method for remediating soil as recited in  
5 claim 25, wherein the redox potential of the slurry when anaerobic is less than about -200 mV.

34. A method for remediating soil as recited in  
claim 25, wherein the inoculum of anaerobic microorganisms  
is comprised of a population of anaerobic microorganisms  
10 grown in nutrient medium, including the nitroaromatic  
compounds present as contaminants in said soil.

35. A method for remediating soil as recited in  
claim 25, wherein the inoculum of anaerobic microorganisms  
is a consortium of multiple species of anaerobic bacteria.

15 36. A method for remediating soil as recited in  
claim 25, wherein at least a portion of said contaminant  
nitroaromatic compounds is degraded to carbon dioxide.

37. A method for remediating soil as recited in  
claim 25, including the steps of placing the  
20 nitroaromatic-contaminated soil in a vessel before adding  
said water to the soil to form said slurry, covering the  
vessel containing the slurry so as to substantially  
exclude gaseous exchange between the slurry and the  
atmosphere, and keeping the slurry contained within the  
25 covered vessel until said anaerobic microorganisms have  
degraded the nitroaromatic compounds in the slurry.

38. A method for treating a soil contaminated  
with aminoaromatic compounds so as to favor mineralization  
of the aminoaromatic compounds in the soil over oxidative  
30 polymerization of the aminoaromatic compounds, the method  
comprising:

(a) adding water to the  
nitroaromatic-contaminated soil to form an aqueous slurry  
of the soil;

35 (b) adding to the slurry an inoculum of  
fermentative amylolytic microorganisms resistant to the  
aminoaromatic compounds in the soil, where the  
fermentative amylolytic microorganisms are selected from

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the group consisting of aerobic and facultative amylolytic microorganisms;

(c) adding to the slurry at least one type of starchy carbohydrate hydrolyzable to constituent sugars by

5 said fermentative amylolytic microorganisms;

(d) maintaining the slurry containing said inoculum of fermentative amylolytic microorganisms and starchy carbohydrate under conditions suitable for growth of said fermentative microorganisms in the slurry,

10 accompanied by hydrolysis of the starchy carbohydrate to constituent sugars and metabolism by said microorganisms of a portion of said sugars, thereby depleting the concentration of dissolved oxygen in the slurry so as to render the slurry anaerobic;

15 (e) after the slurry has been rendered anaerobic, adding to the slurry an inoculum of anaerobic microorganisms capable of utilizing as a carbon and energy source said sugars formed in the slurry by the amylolytic microorganisms and capable of degrading said aminoaromatic compounds; and

(f) maintaining anaerobic conditions in the slurry for a time sufficient for the anaerobic microorganisms to degrade the contaminant aminoaromatic compounds.

25 39. A method for treating soil as recited in claim 38, wherein the water is added to the soil in a volume sufficient to saturate the water-holding capacity of the soil.

40. A method for treating soil as recited in

30 claim 38, including the step of adding a source of metabolizable nitrogen to the slurry to support the growth of the microorganisms inoculated into the slurry.

41. A method for treating soil as recited in claim 38, wherein the slurry is maintained at a pH within 35 a range of about 6 to 8.

42. A method for treating soil as recited in claim 38, wherein the slurry is maintained at a

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temperature within a range of about 10°C to less than about 40°C.

43. A method for treating soil as recited in claim 38, wherein the redox potential of the slurry when 5 anaerobic is less than about -200 mV.

44. A method for treating soil as recited in claim 38, wherein the inoculum of fermentative amylolytic microorganisms is comprised of a population of amylolytic microorganisms grown in aerobic nutrient medium including 10 the aminoaromatic compounds present as contaminants in said soil.

45. A method for treating soil as recited in claim 38, wherein the inoculum of anaerobic microorganisms is comprised of a population of anaerobic microorganisms 15 grown in nutrient medium including the aminoaromatic compounds present as contaminants in said soil.

46. A method for treating soil as recited in claim 38, wherein the inoculum of anaerobic microorganisms is a consortium of multiple species of anaerobic bacteria.

20 47. A method for treating soil as recited in claim 38, including the steps of placing the aminoaromatic-contaminated soil in a vessel before adding said water to the soil to form said slurry, covering the vessel containing the slurry so as to substantially 25 exclude gaseous exchange between the slurry and the atmosphere, and keeping the slurry contained in the covered vessel until said anaerobic microorganisms have degraded the aminoaromatic compounds in the slurry.

48. A two-stage process for treating a fluid 30 medium contaminated with nitroaromatic compounds so as to at least partially degrade and thereby reduce the concentrations of the nitroaromatic compounds in the fluid medium, the process comprising:

(a) an aerobic stage wherein are added to the 35 fluid medium an inoculum of fermentative microorganisms resistant to the concentration of nitroaromatic compounds present in the fluid medium where the fermentative microorganisms are selected from the group consisting of

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aerobic and facultative microorganisms, at least one type of carbohydrate fermentable by said fermentative microorganisms, and a source of metabolizable nitrogen, after which addition the fluid medium is maintained under

5 conditions suitable for growth of the fermentative microorganisms in the fluid medium, accompanied by fermentation by said microorganisms of a portion of said carbohydrate, thereby depleting the concentration of dissolved oxygen in the fluid medium so as to render the

10 fluid medium anaerobic; and

(b) an anaerobic stage after the aerobic stage, in which anaerobic stage is added to the fluid medium an inoculum of anaerobic microorganisms capable of utilizing said carbohydrate remaining from the aerobic stage as a carbon and energy source and of degrading said nitroaromatic compounds, after which addition the fluid medium is maintained in an anaerobic condition for a time sufficient for the anaerobic microorganisms to degrade the contaminant nitroaromatic compounds in the fluid medium.

20 49. A two-stage process as recited in claim 48, wherein the fluid medium is maintained at a pH within a range of about 6 to 8.

50. A two-stage process as recited in claim 48, wherein the fluid medium is maintained at a temperature

25 within a range of about 10°C to less than about 40°C.

51. A two-stage process as recited in claim 48, wherein the source of metabolizable nitrogen is selected from the group consisting of ammonium compounds and amino compounds.

30 52. A two-stage process as recited in claim 48, wherein the inoculum of fermentative microorganisms is comprised of a population of microorganisms grown in aerobic nutrient medium including the nitroaromatic compounds present as contaminants in said fluid medium.

35 53. A two-stage process as recited in claim 48, wherein the carbohydrate comprises a starch.

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54. A two-stage process as recited in claim 53, wherein the inoculum of fermentative microorganisms includes amylolytic microorganisms.

55. A two-stage process as recited in claim 48, 5 wherein the inoculum of anaerobic microorganisms is comprised of a population of anaerobic microorganisms grown in nutrient medium including the nitroaromatic compounds present as contaminants in said fluid medium.

56. A two-stage process as recited in claim 48, 10 wherein the inoculum of anaerobic microorganisms is a consortium of multiple species of anaerobic bacteria.

57. A method for at least partially degrading nitroaromatic compounds present as contaminants in a fluid medium to reduce the concentrations of the nitroaromatic 15 compounds in the fluid medium, the method comprising:

(a) sequestering the fluid medium from the atmosphere so as to prevent gaseous exchange between the fluid medium and the atmosphere;

(b) rendering the fluid medium anaerobic;

20 (c) adding to the fluid medium an inoculum of anaerobic microorganisms capable of degrading the nitroaromatic compounds in the fluid medium;

(d) adding to the fluid medium metabolizable sources of carbon, energy, and nitrogen for the anaerobic 25 microorganisms inoculated into the fluid medium;

(e) maintaining the fluid medium in an anaerobic condition for a time sufficient for the anaerobic microorganisms to degrade the contaminant nitroaromatic compounds in the fluid medium.

30 58. A method for degrading nitroaromatic compounds in a fluid medium as recited in claim 57, wherein the fluid medium is rendered anaerobic by purging oxygen from the fluid medium.

35 59. A method for degrading nitroaromatic compounds in a fluid medium as recited in claim 57, wherein the fluid medium is rendered anaerobic by adding to the fluid medium an oxygen-scavenging agent.

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60. A method for degrading nitroaromatic compounds in a fluid medium as recited in claim 57, wherein the fluid medium is sequestered from the atmosphere by placing the fluid medium in a vessel for 5 containing the fluid medium and covering the vessel.

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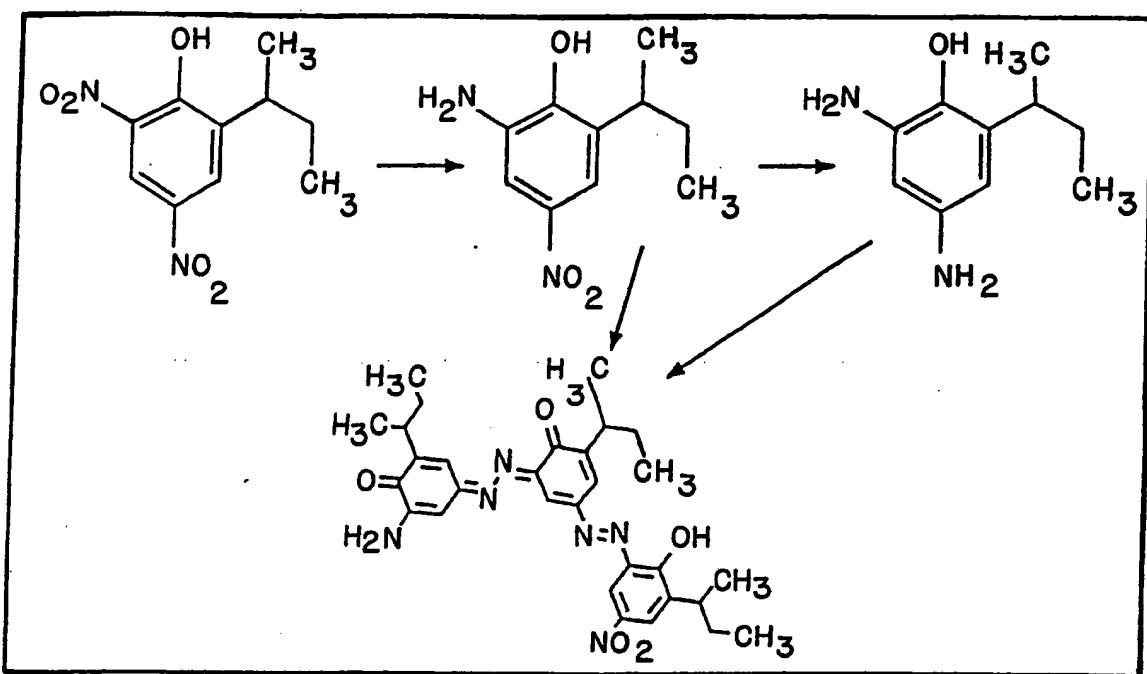


FIG. 1

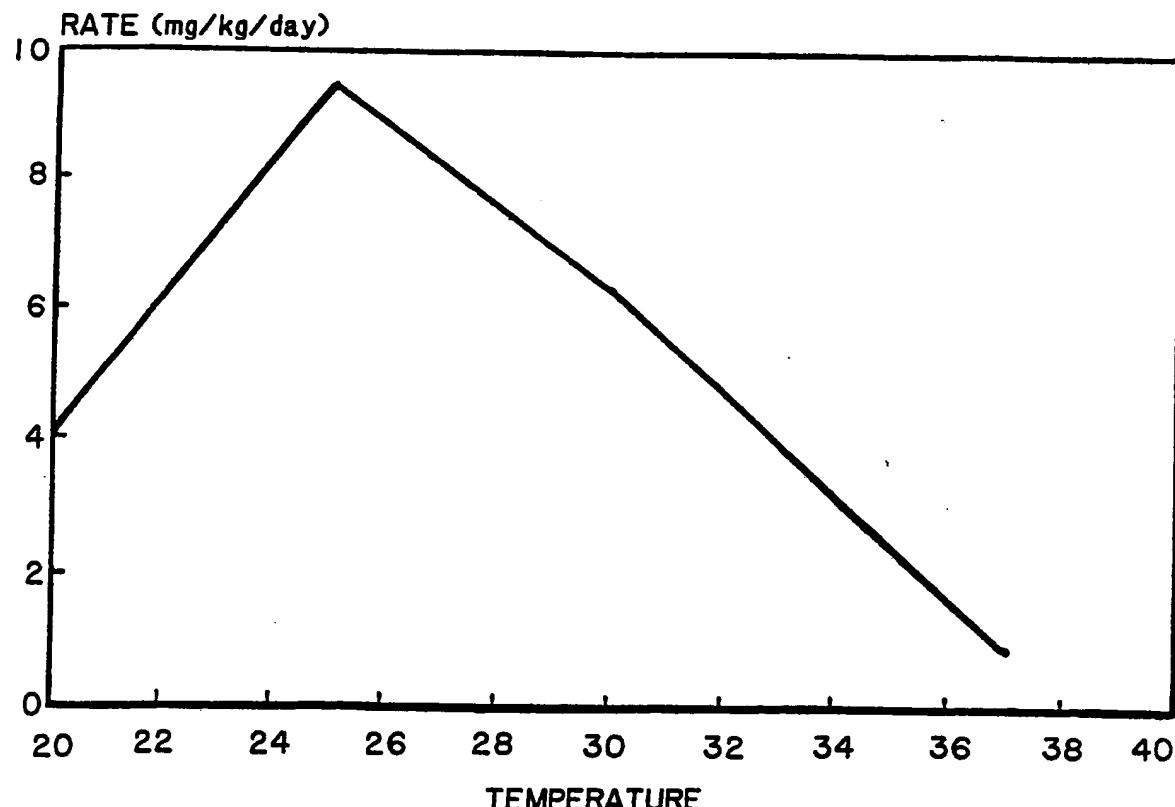


FIG. 2

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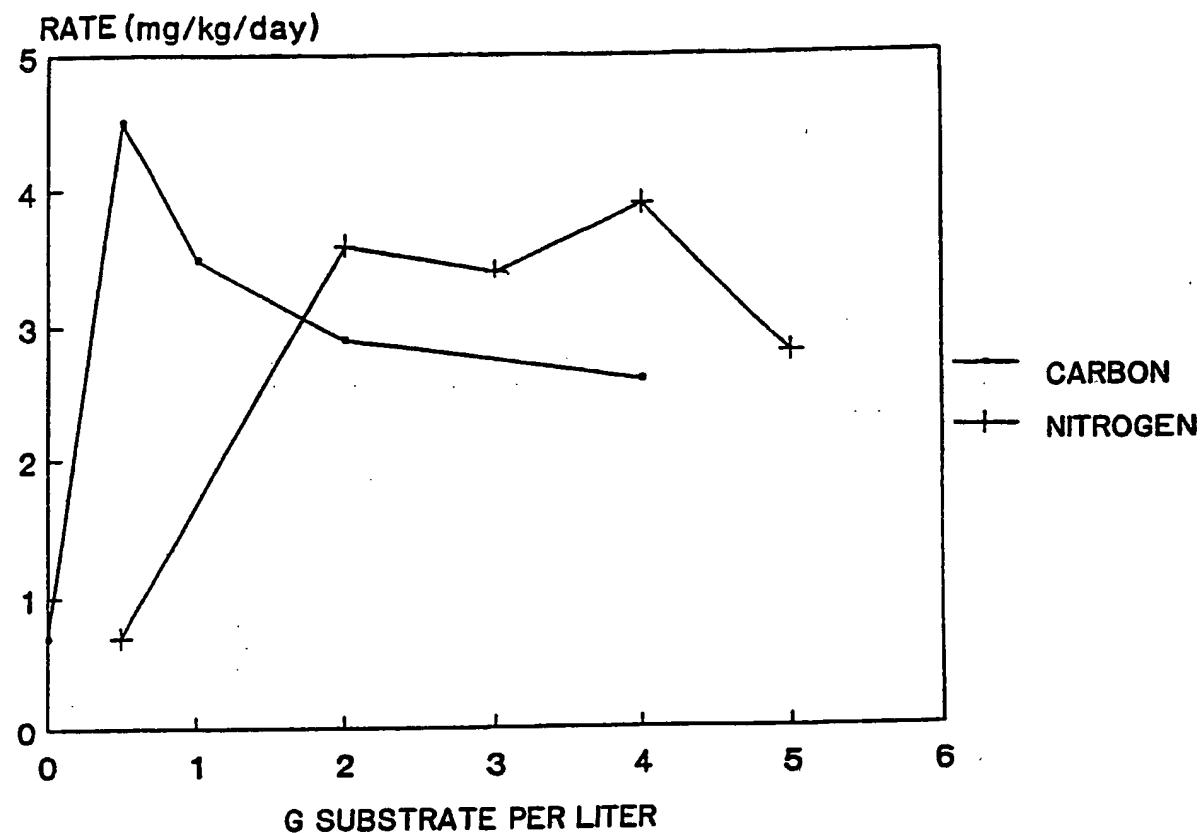


FIG. 3

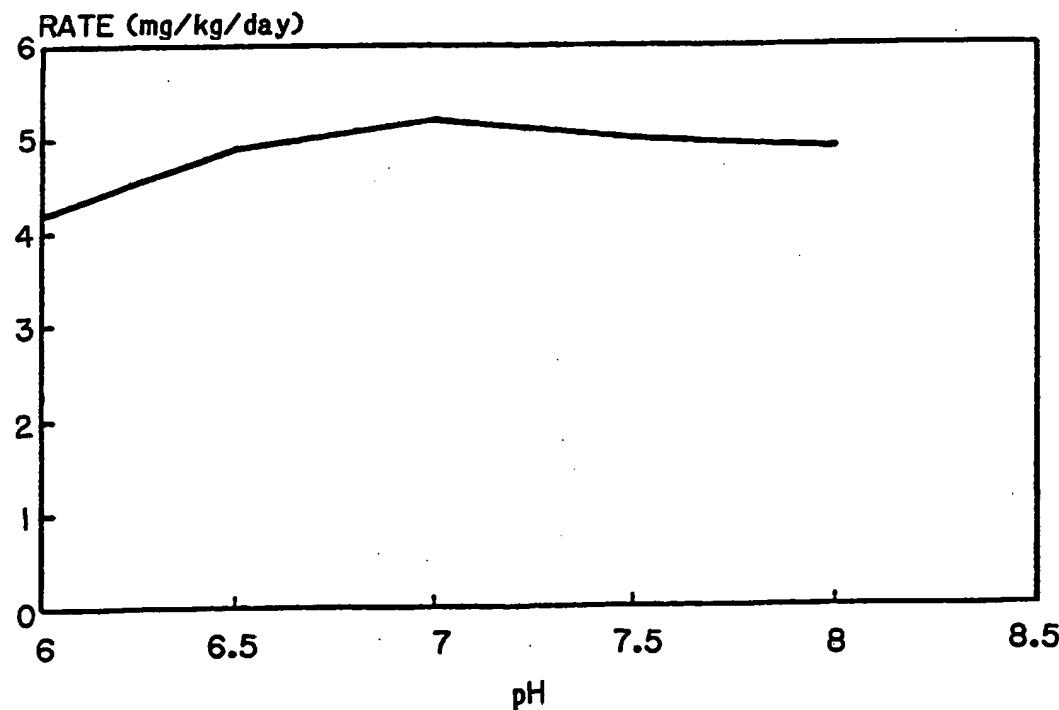


FIG. 4

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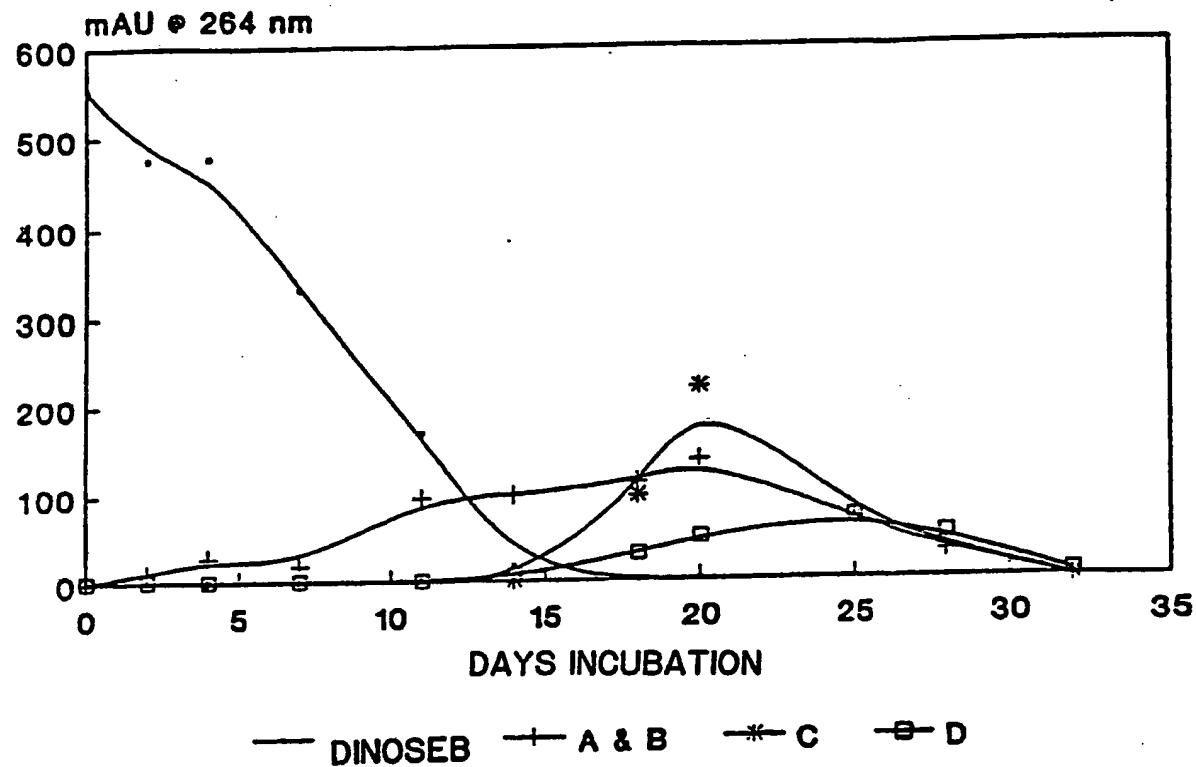


FIG. 5

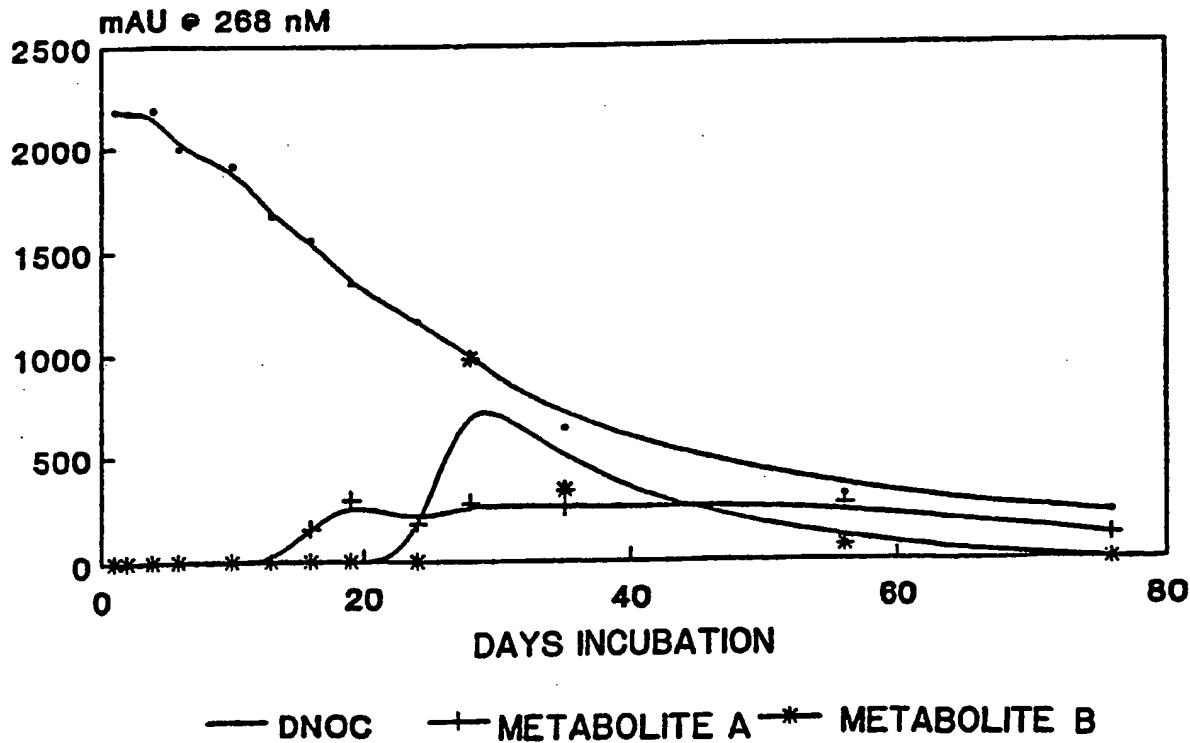


FIG. 6

SUBSTITUTE SHEET

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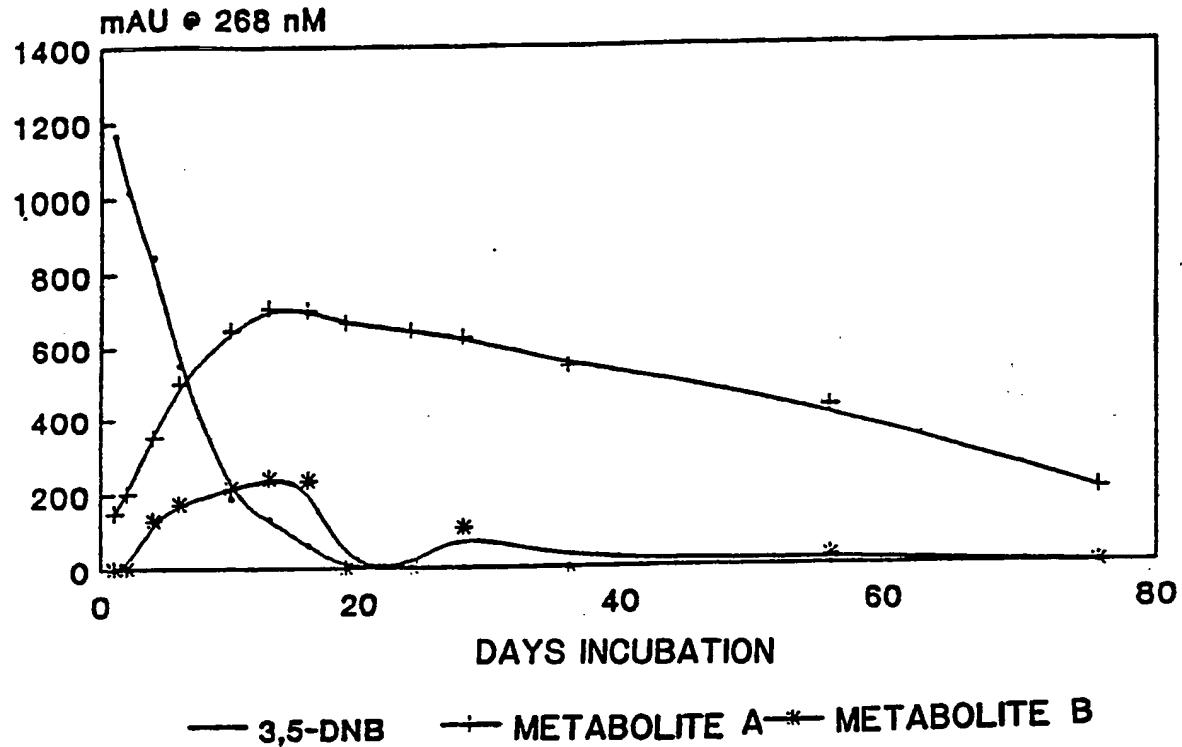


FIG. 7

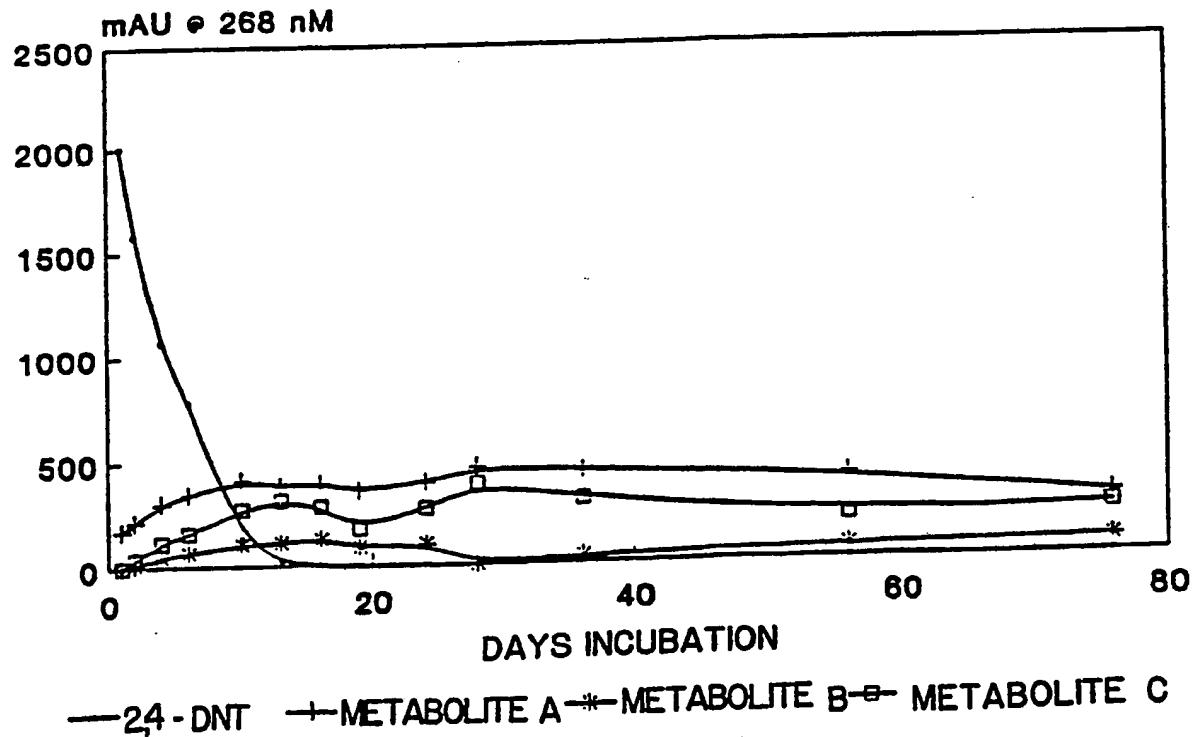


FIG. 8

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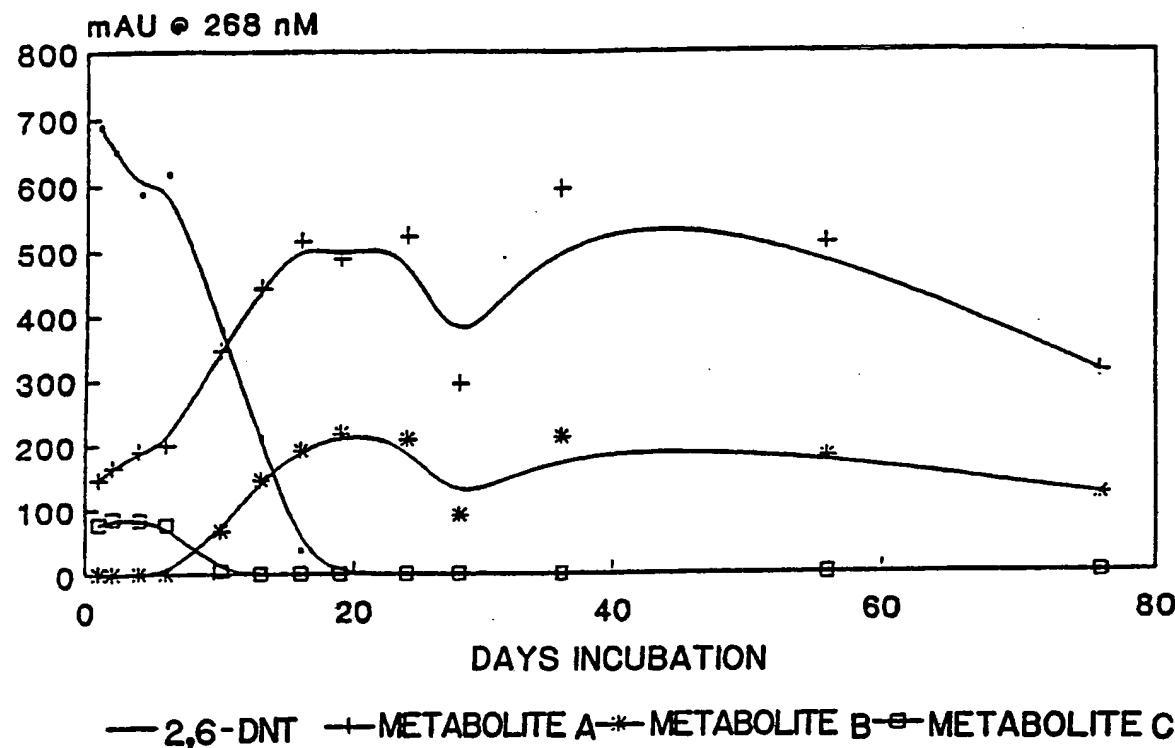


FIG. 9

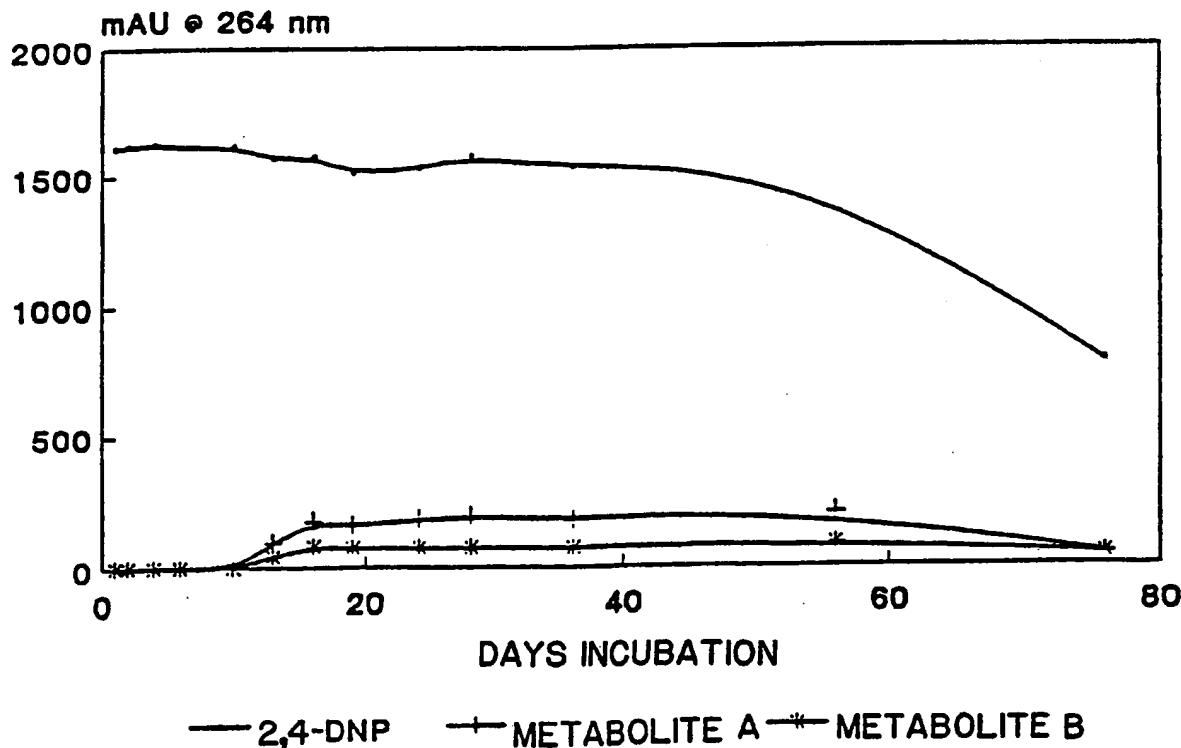


FIG. 10

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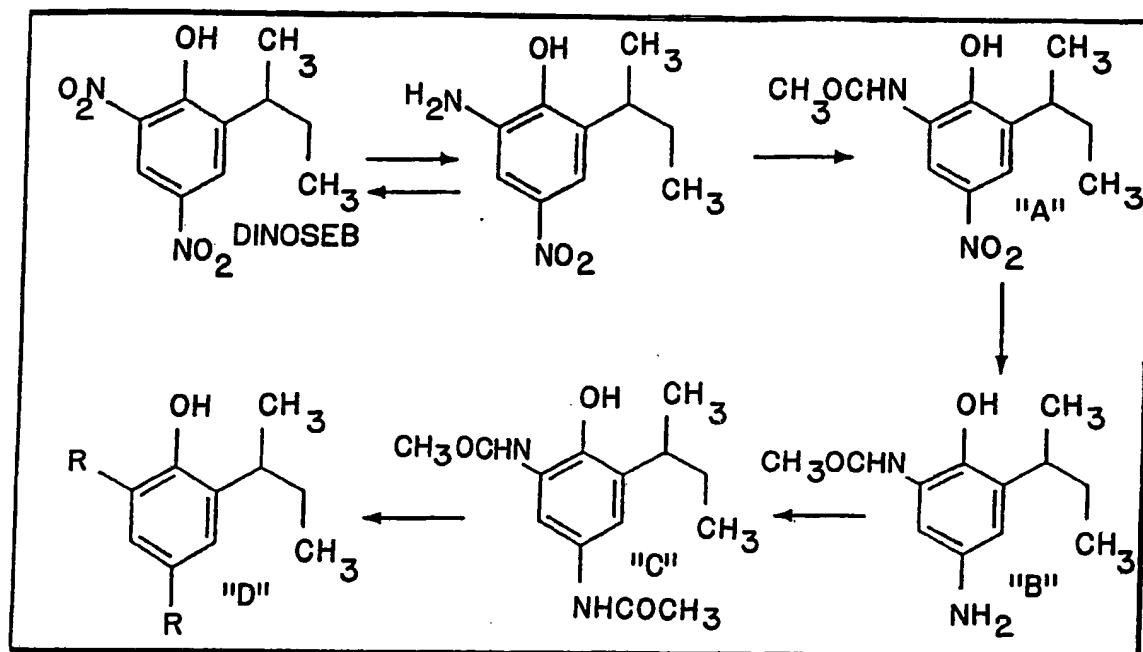


FIG. 11

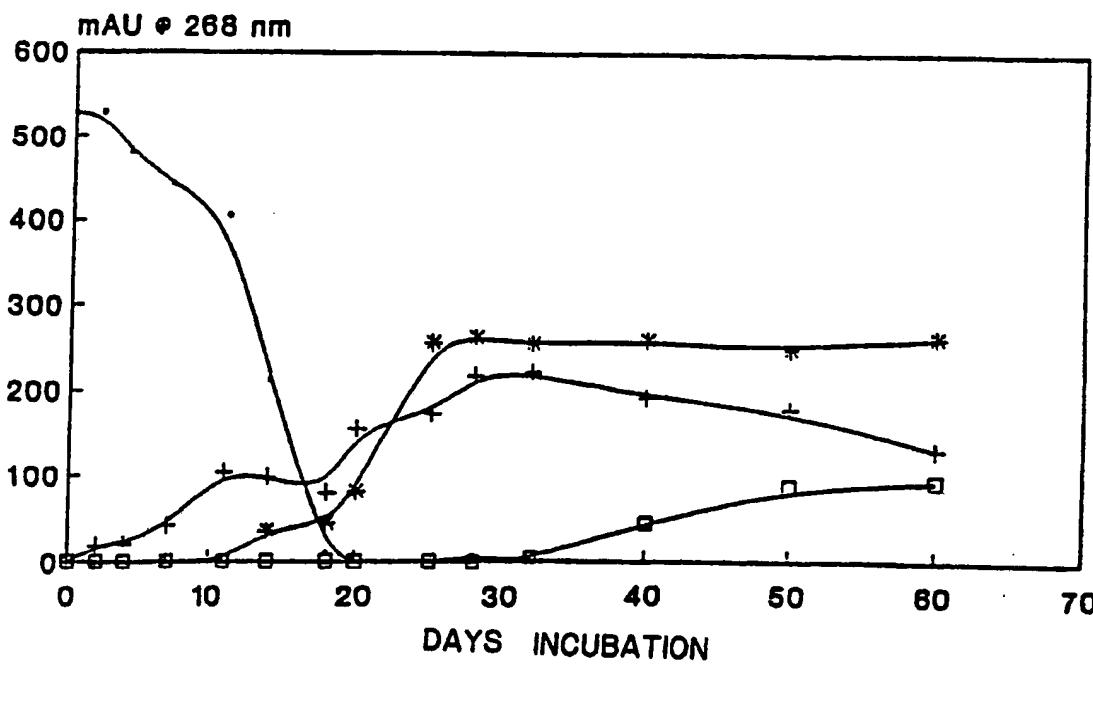


FIG. 12

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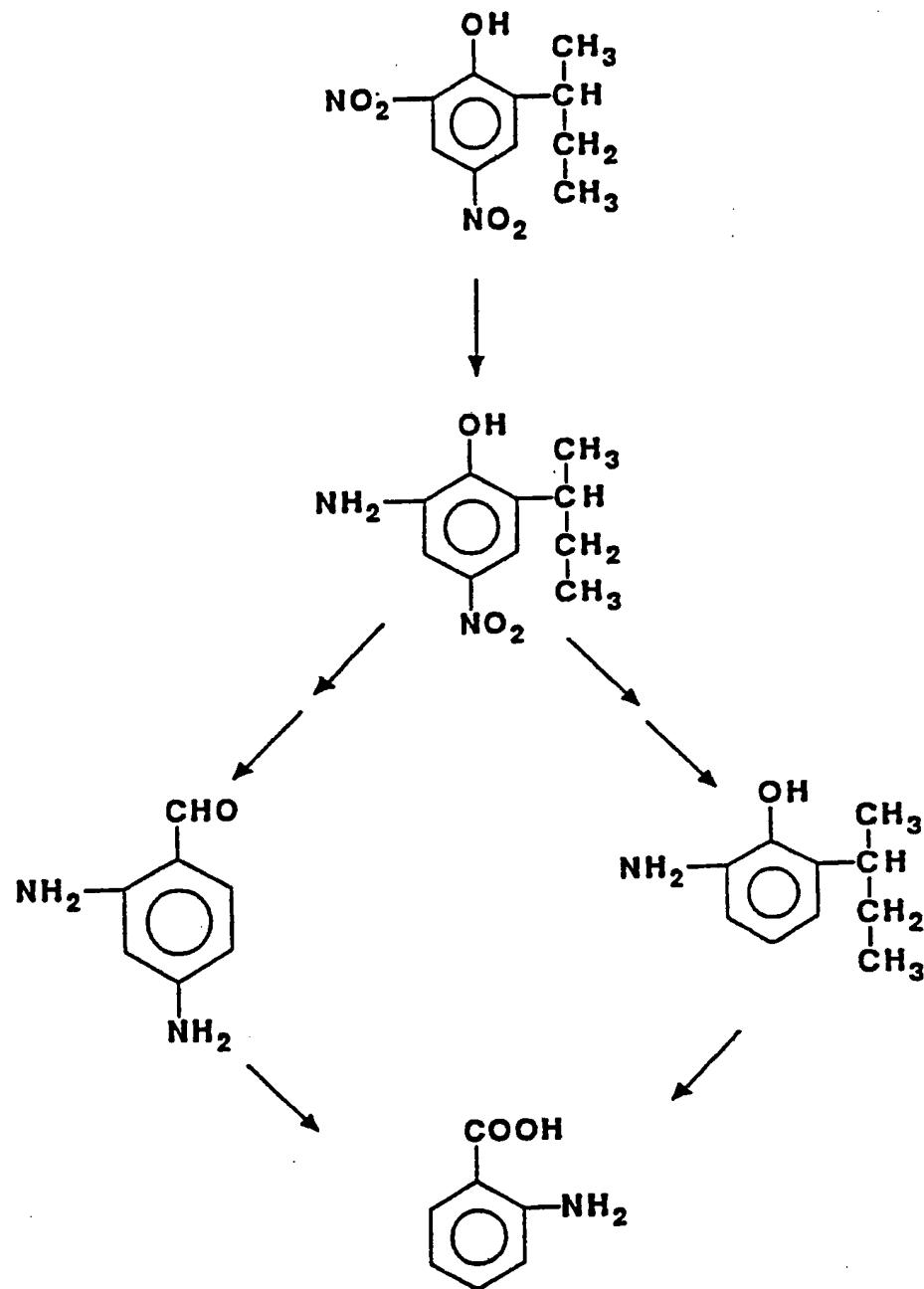
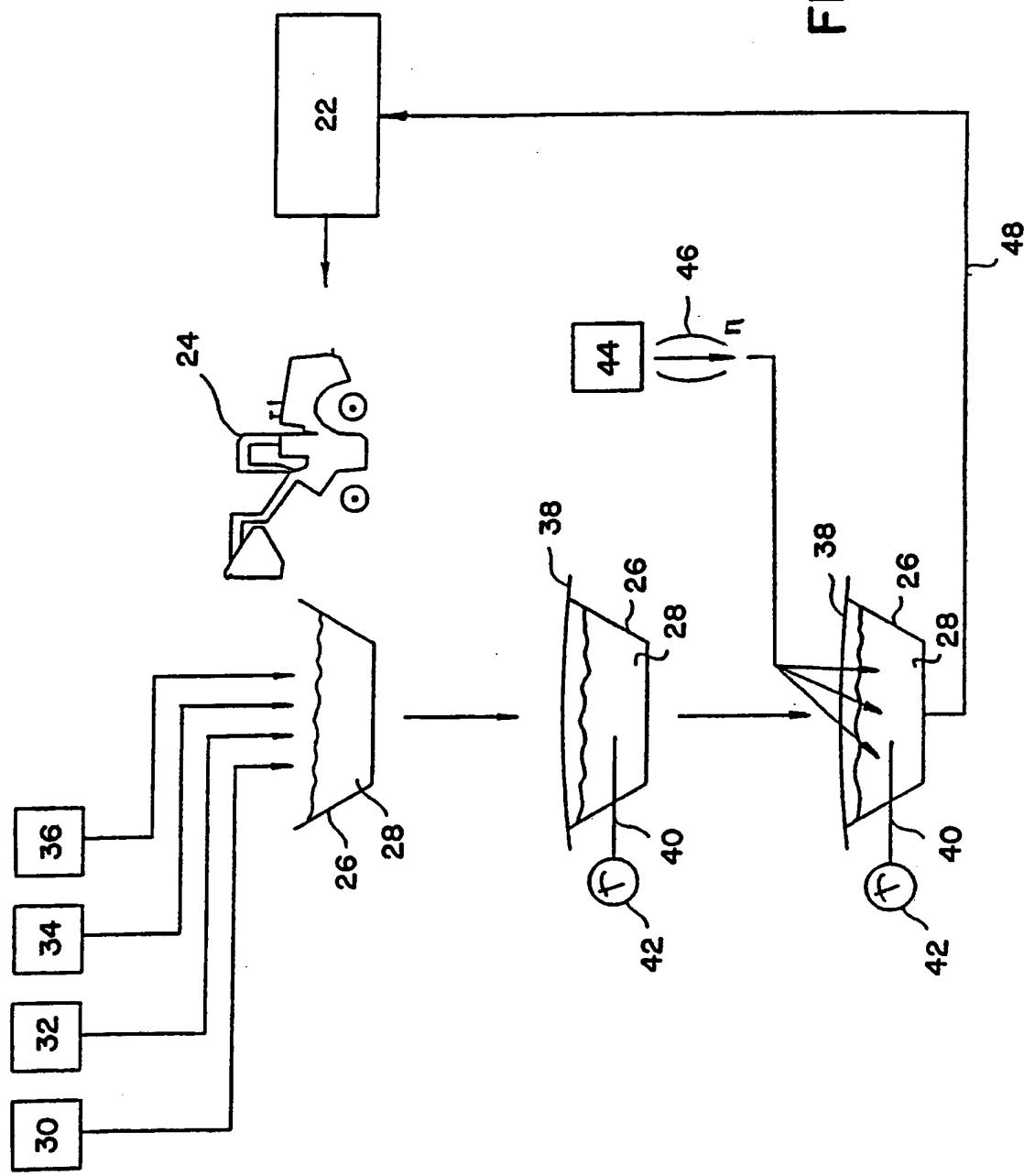


FIG. 13

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FIG. 14



# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/02416

## I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) \*

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C05F 11/08; C05G 3/00; C02F 3/00  
US. CL: 71/9,10,903; 210/610,611

## II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
US	71/8,9,10,903 210/610,611

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched \*

## III. DOCUMENTS CONSIDERED TO BE RELEVANT \*

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
L,A	US, A, 4,968,427 (Glanser et al) 06 November 1990 See entire document	1-24,48-60
A	US, A 4,826,601 (Spratt et al) 02 May 1989 See entire document	1-24,48-60
Y	K.BRAUN et al "Anaerobic Digestion of 2-Aminobenzoate (Anthranilic Acid) by Denitrifying Bacteria", Published July, 1984, by American Society for Microbiology, see pages 102-107	25-47
Y	T.W.FEDERLE, "Mineralization of Monosubstituted aromatic compounds in unsaturated and saturated subsurface soils", published 1988 by Canadian Journal of Microbiology, see pages 1037-1042	25-47

- \* Special categories of cited documents: 10
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search

24 June 1991

International Searching Authority

ISA/US

Date of Mailing of this International Search Report

16 AUG 1991

Signature of Authorized Officer *Nguyen*  
Ferris H. Landert, NATIONAL DIVISION  
JULY 1991  
Ferris H. Landert, NATIONAL DIVISION

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V.  OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE<sup>1</sup>

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.  Claim numbers \_\_\_\_\_, because they relate to subject matter<sup>12</sup> not required to be searched by this Authority, namely:

2.  Claim numbers \_\_\_\_\_, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out<sup>13</sup>, specifically:

3.  Claim numbers \_\_\_\_\_, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI.  OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING<sup>2</sup>

This International Searching Authority found multiple inventions in this International application as follows:

I. Claims 1-24 and 48-60, drawn to a method of degrading nitroaromatic compounds in fluids.

II. Claims 25-47, drawn to a method of remediating soils.

1.  As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4.  As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- The additional search fees were accompanied by applicant's protest.  
 No protest accompanied the payment of additional search fees.

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